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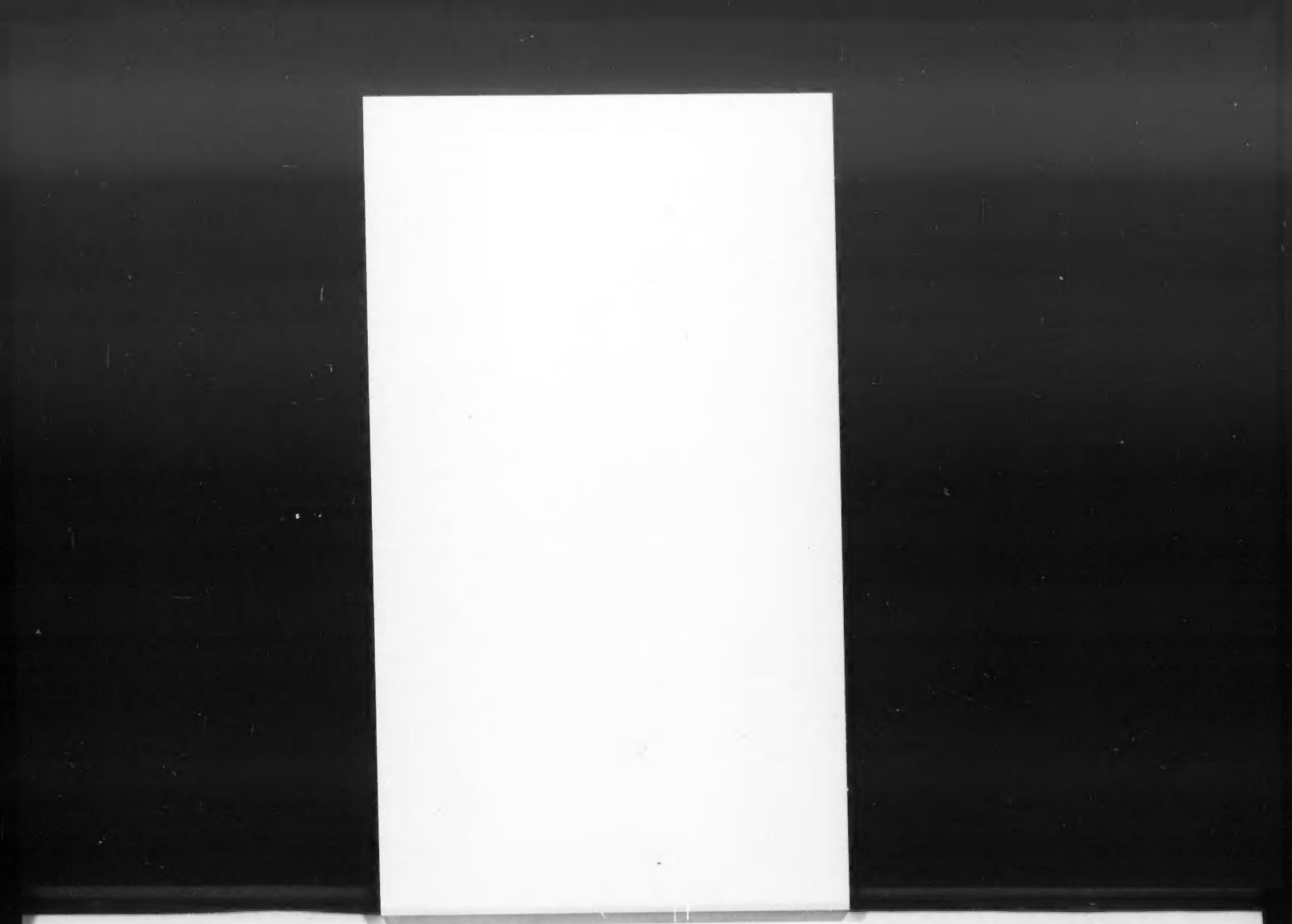
ERRATA

Hypervitaminosis A.
By T. Moore & Y. L. Wang
Volume 39 (1945), No. 3, p. 226, col. 2, line 7 from bottom

for vitamin A *read* vitamin C.

The Chemical Estimation of Vitamin E in Vegetable Oils.
By J. Tošić & T. Moore
Volume 39 (1945), No. 5, p. 504, col. 2, line 2 from bottom

for 5 ml. *read* 15 ml.



The Influence of Phytic Acid on the Absorption of Calcium and Phosphorus

I. IN DOGS

By E. HOFF-JØRGENSEN, *Biochemical Institute, University of Copenhagen, Denmark*

(*Received* 31 *August* 1945)

In 1920 Mellanby demonstrated that increasing the amount of cereals in diets deficient in vitamin D leads to increased intensity of rickets. Steenbock, Black & Thomas (1930) drew attention to the possibility that the organic phosphorus compound present in cereals may not be physiologically equivalent to inorganic phosphates added to the diet. Bruce & Callow (1934) established the fact that in rats the P of phytic acid is not so easily available as that of sodium phosphate.

Since phytic acid precipitates calcium (Starkenstein (1910)), Bruce & Callow (1934) suggested that in diets with a natural Ca/P ratio the main action of phytic acid might be that it renders Ca unavailable. In dogs the rachitogenic effect of both sodium phytate and of cereals is antagonized by addition of extra Ca to the diet (Harrison & Mellanby, 1939).

By determinations of the Ca and P balance in dogs we have attempted to investigate what part phytic acid plays in the absorption of Ca and P. The results of these experiments are now reported and the results of similar investigations on infants and children will follow shortly.

METHODS

General. The experiments were made on two male puppies (nos. 5 and 6) of the same litter, born 25 September 1944. From the 30th day of their lives each animal was given the following diet of semolina-milk gruel each day: 250 g. of whole milk; 15 g. of semolina; 15 g. of sugar; 25 g. of liver paste; 2·5 g. of sawdust; 2·5 g. of dried yeast; 0·05 g. of $FeSO_4.7H_2O$; 3000 i.u. of vitamin A; 400 i.u. of vitamin D_2; 2·860 g. of $CaHPO_4.2H_2O$; 0·668 g. of $NaH_2PO_4.H_2O$ and distilled water *ad libitum*.

This diet provides 1 g. of Ca/day (0·298 g. from the milk, 0·008 g. from the other foodstuffs and 0·694 g. from $CaHPO_4.2H_2O$) and 1 g. of P/day (0·245 g. from the milk, 0·065 g. from the other foodstuffs, 0·540 g. from $CaHPO_4.2H_2O$ and 0·150 g. from $NaH_2PO_4.H_2O$).

During the periods in which the effect of phytic acid was investigated, the diet was altered: instead of calcium phosphate 3·235 g. of pentacalcium phytate (containing 0·694 g. of Ca) and 2·905 g. of sodium phytate ($Na_{12}C_6H_6O_{24}P_6.38H_2O$) in neutral solution was mixed with the gruel. This amount of sodium phytate is exactly sufficient to precipitate the 0·306 g. of Ca in the milk and the other foodstuffs as pentacalcium phytate. The total amount of Ca in this diet is therefore also 1 g./day and the total amount of phytic acid is equivalent to the Ca content. In order that the content of non-phytic P of the diet in these periods should also total 1 g./day, 0·690 g. of P was added as a mixture of equal parts of primary and secondary sodium phosphate. As the dogs grew older the diet was supplemented with sugar (maximum 35 g./day) and rice starch (maximum 30 g./day). The increase in Ca content of the diet due to the supplementary food, less than 0·001 g./day, has not been considered. On the other hand, the increase in the P content, a maximum of 0·045 g./day, was corrected by lowering the amount of sodium phosphate. To ensure that the dogs ate their full ration every day, it was necessary to keep them slightly undernourished. They were therefore rather thin during the whole experiment.

At the age of about 140 days the dogs developed distemper and the experiment had to be broken off. Dog no. 6 recovered fairly quickly, but no. 5 was ill for about a month, and after it had otherwise recovered, it still had a slight paralysis of the hind legs. From the 142–150th day they were given a daily injection of 2 ml. of a preparation of 'vitamin B' (*Becoplex* from the firm *Ferrosan*). For a few days the dogs could not eat their ration, but even during their severest illness the administration of Ca and P was as far as possible 1 g./day. When phytate was added to the diet, carmine was also given, in order to decide which stools belonged to the phytate periods. The change in weight with age of the 2 dogs was:

Age in days ...	30	62	90	121	142	175	210	242	262
Dog no. 5: weight in g.	910	1625	2400	3125	3320	3560	4010	4490	4630
Dog no. 6: weight in g.	980	1800	2510	3440	3770	4200	4650	5100	5270

Methods of estimation. Ca was determined after dry ashing by the method of Larson & Greenberg (1938), total P after wet ashing by the method of Grænsholm (1935), and phytate by the method of McCance & Widdowson (1935). To obtain complete precipitation of the ferric phytate it was necessary to boil for 30 min. and let the solutions stand for at least 12 hr.

RESULTS

During the periods in which phytate was administered the retention of Ca decreased; the excretion of Ca in the urine, however, was not definitely

Table 1. *The influence of phytic acid on the retention of calcium and phosphorus*

Age (days)	Intake (mg.) of Ca	Intake (mg.) of Total P	Intake (mg.) of Phytate P	Excretion (mg.) of P in urine	Excretion (mg.) of P in faeces	Excretion (mg.) of Ca in urine	Excretion (mg.) of Ca in faeces	Excretion (mg.) of Phytate P in faeces	Retention (mg.) of Ca	Retention (mg.) of P
Dog no 5:										
31–36	5000*	5000	0	1770	1930	42	3860	0	1098	1300
38–43	5000†	9655	4655	2210	5800	36	4130	2840	834	1645
45–50	5000*	5000	0	1540	2255	30	3520	0	1205	1205
74–79	5000*	5000	0	1320	2500	64	3515	0	1421	1180
81–86	5000†	9655	4655	2465	5375	32	4390	2480	578	1875
88–93	5000*	5000	0	1640	2085	41	3710	0	1249	1275
119–124	5000*	5000	0	1430	2110	105	3375	0	1520	1460
126–131	5000†	9655	4655	2045	5820	56	4540	2160	404	1790
133–138	5000*	5000	0	1390	2600	52	3745	0	1203	1010
194–199	5000*	5000	0	1515	2605	80	3930	0	990	880
202–207	5000†	9655	4655	2720	5130	42	5275	2210	−317	1805
210–215	5000*	5000	0	1275	2585	65	3875	0	1060	1140
236–241	5000*	5000	0	1600	2480	38	4042	0	730	920
243–248	5000†	9655	4655	2585	5390	42	5240	2270	−282	1680
250–255	5000*	5000	0	1460	2600	65	4195	0	800	940
Dog no. 6:										
31–36	5000*	5000	0	1525	2275	20	3655	0	1325	1200
38–43	5000†	9655	4655	2205	5700	16	4275	2220	709	1750
45–50	5000*	5000	0	1430	2625	27	3775	0	1198	945
74–79	5000*	5000	0	1360	2125	30	3350	0	1620	1515
81–86	5000†	9655	4655	2080	5750	30	4240	2865	730	1825
88–93	5000*	5000	0	1640	2100	38	3445	0	1517	1260
119–124	5000*	5000	0	1460	2115	15	3720	0	1265	1425
126–131	5000†	9655	4655	2195	5545	16	4475	3020	509	1915
133–138	5000*	5000	0	1230	2545	42	3490	0	1468	1225
154–159	5000*	5000	0	1065	3175	46	4135	0	819	760
161–166	5000†	9655	4655	2385	5090	12	4530	2125	458	2180
168–173	5000*	5000	0	1745	2170	28	3645	0	1327	1085
194–199	5000*	5000	0	1260	2860	60	4000	0	940	880
203–207	5000†	9655	4655	2420	5355	32	5180	2265	−212	1880
209–214	5000*	5000	0	1120	3035	28	4140	0	832	845
236–241	5000*	5000	0	1535	2280	43	4090	0	867	1185
243–248	5000†	9655	4655	2415	5520	25	5355	3420	−360	1720
250–255	5000*	5000	0	1605	2570	28	4030	0	942	825

* 1·490 g. Ca derived from milk, 0·040 g. Ca derived from other foodstuffs and 3·470 g. Ca derived from $CaHPO_4.2H_2O$.
† 1·490 g. Ca derived from milk, 0·040 g. Ca derived from other foodstuffs and 3·470 g. Ca derived from calcium phytate ($C_6H_8O_{24}P_6Ca_5$).

Table 2. *The influence of oxalic acid on the retention of calcium and phosphorus*

Dog no.	Age (days)	Intake (mg.) of Ca	Intake (mg.) of P	Intake (mg.) of Calcium oxalate	Intake (mg.) of Sodium oxalate	Excretion (mg.) of P in urine	Excretion (mg.) of P in faeces	Excretion (mg.) of Ca in urine	Excretion (mg.) of Ca in faeces	Retention (mg.) of Ca	Retention (mg.) of P
5	257–262	5000	5000	11,120	5121	2240	1330	20	5860	−880	1430
6	257–262	5000	5000	11,120	5121	2435	945	36	5975	−1011	1620

Table 3. *The influence of oxalic acid and phytic acid on the absorption of phosphorus*

Age (days)	Intake (mg.) of Ca	Intake (mg.) of Total P	Intake (mg.) of Calcium oxalate	Intake (mg.) of Sodium oxalate	Intake (mg.) of Phytic acid P	Absorption* of P (mg.) Dog no. 5	Absorption* of P (mg.) Dog no. 6
236–241	5000	5000	0	0	0	2520	2720
250–255	5000	5000	0	0	0	2400	2430
257–262	5000	5000	11,120	5121	0	3670	4065
243–248	5000	9655	0	0	4655	4265	4135

* Difference between intake and faecal output.

influenced, not even in periods with a negative Ca balance (Table 1). The decrease in the absorption of Ca was far more pronounced when the dogs received phytate (Fig. 1), and their ability to absorb Ca when in a low concentration in the intestine seems to be very much reduced as they grow older.

Table 1 also shows that both absorption and retention of P is greater when phytate is administered than in the phytate-free periods. This fact may either be due to the phytate being hydrolyzed in the intestine and the split phosphate then being absorbed, or to the Ca ions being bound to the phytate, which would cause an increased concentration of P ions. The testing of these possibilities might be made with phytate with labelled P atoms. At present this is hardly possible. Another way of

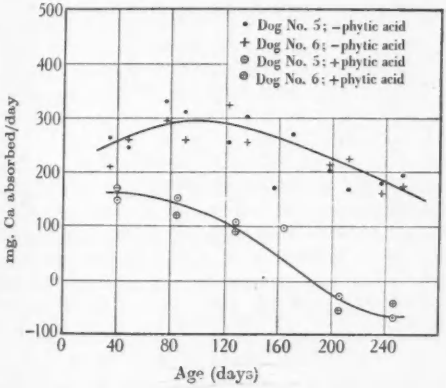


Fig. 1. Changes in Ca absorption in dogs. Daily intake of Ca: 0·298 g. from milk, 0·008 g. from other foodstuffs and 0·694 g. from $CaHPO_4.2H_2O$ or pentacalcium phytate. In the phytate periods sodium phytate sufficient to precipitate the Ca content in the milk and the other foodstuffs as pentacalcium phytate was added to the diet.

solving the problem was attempted. In experiments similar to the above sodium oxalate and calcium oxalate were added to the diet instead of sodium phytate and calcium phytate. The feeding of sodium oxalate and calcium oxalate to the dogs led to a decreased absorption of Ca—but an increased absorption of P (Table 3). Calculation of the absorption of P for the four last experimental periods (Table 3) shows that the increase in phosphate absorption is of the same order of magnitude whether the Ca of the food is present as oxalate or as phytate. It may, therefore, be supposed that in spite of the fact that about half the ingested phytate is hydrolyzed, the phosphate split off will only be absorbed to a small degree, probably because the hydrolysis is due to bacteria and therefore mainly takes place in the colon.

DISCUSSION

In accordance with the result of Harrison & Mellanby (1939) that the rachitogenic action of phytic acid can be antagonized by adding extra Ca to the diet, we have found that the addition of phytic acid to the diet of dogs entails a diminution both in absorption as well as in retention of Ca. The decrease is, however, moderate when the puppies are less than 2 months old, but becomes much more pronounced when the animals grow older. On a diet containing equivalent amounts of Ca and phytic acid (i.e. when Ca/phytic acid P = 5/6) the Ca balance becomes negative at about the time of maturity, that is, when the animals are about 6 months old, and while they are still growing rapidly. In 1944, Hoff-Jørgensen demonstrated that pentacalcium phytate ($C_6H_8O_{24}P_6Ca_5$) is precipitated in the pH range of 5–7. He also showed that the solubility of pentacalcium phytate is greater than that of hydroxy-apatite ($Ca_5(PO_4)_3.OH$), but less than that of secondary calcium phosphate. The Ca salt of phytic acid is precipitated instantly, whilst secondary calcium phosphate precipitates slowly. The difference in the solubility of the secondary phosphate and the phytate is not great enough to explain why Ca absorption is so much impaired when Ca is administered together with phytate. The difference in speed of precipitation, however, might explain the difference in the absorption of Ca present as phosphate and as phytate. In accord with McCance & Widdowson (1942) we found that about 50 % of the phytic acid in the diet is hydrolyzed in the intestine, and that the addition of phytic acid to the diet increases the absorption of phosphate. McCance & Widdowson (1942) think that the increased phosphate-absorption is due to the absorption of a great part of the hydrolyzed phytic acid P. However, our experiments show that the phosphate absorption is almost equally great when oxalate is added to the diet instead of phytic acid. It is therefore more likely that the increased phosphate absorption is due to the fact that phytic acid, like oxalate, precipitates the Ca, the concentration of phosphate ions being thereby increased, since $[Ca^{++}].[HPO_4^{--}] = K_s$. It is furthermore probable that the hydrolysis of phytic acid is mainly due to bacteria and takes place in the colon, from which no phosphate is absorbed.

SUMMARY

1. In puppies replacement of the calcium phosphate of the diet by pentacalcium phytate and the addition of sodium phytate sufficient to precipitate dietary Ca of foodstuffs led to decreased retention of Ca and increased retention of P.

2. A decline in Ca retention occurred with age.

3. Administration of phytate and oxalate increased the absorption of P in nearly the same degree.

The above work is part of the programme of the Cereal Committee of the Academy of Technical Sciences, Copenhagen.

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Studies on Cholinesterase

5. THE SELECTIVE INHIBITION OF PSEUDO-CHOLINESTERASE *IN VIVO*

By ROSEMARY D. HAWKINS and JOSEPHINE M. GUNTER, *Banting and Best Department of Medical Research, Banting Institute, University of Toronto, Canada*

(*Received* 13 *November* 1945)

Mendel & Rudney (1943*a*) showed that two cholinesterases exist in the animal body: a specific cholinesterase acting exclusively on choline esters, and a non-specific enzyme capable of hydrolyzing not only esters of choline but a variety of non-choline esters as well. Mendel & Rudney named the non-specific cholinesterase pseudo-cholinesterase because they doubted the possibility that this enzyme could play an essential part in the hydrolysis of acetylcholine *in vivo*. This assumption has gained further support from the results of recent experiments: (*a*) the non-specific cholinesterase is absent from brain tissue throughout the animal kingdom (Mendel & Rudney, 1943*b*); (*b*) it is not present in the blood and tissues of ox and sheep (Mendel, Mundell & Rudney, 1943; Gunter, unpublished); (*c*) it persists in the superior cervical ganglion of the cat when total degeneration of the preganglionic nerve endings has been effected, whereas the specific cholinesterase disappears completely under these conditions (Sawyer & Hollinshead, 1945).

In spite of the above-mentioned indications, definite proof that this enzyme is not essential to the process of nerve impulse transmission is still lacking. The experiments to be reported here were designed to study this problem.

In the course of investigations on the inhibitory action of certain prostigmin analogues on the specific and on the non-specific cholinesterase *in vitro*, the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl) trimethylammonium bromide (Hoffmann-LaRoche Nu-683) was examined. This compound, in concentrations which had little or no effect on the activity of the specific enzyme, was found to inhibit the non-specific enzyme almost completely. It seemed, therefore, that if a dose of this inhibitor which would suppress the activity of the non-specific cholinesterase *in vivo* elicited no symptoms indicative of acetylcholine poisoning, proof that this enzyme is not essential to the hydrolysis of acetylcholine *in vivo* would be established.

MATERIALS AND METHODS

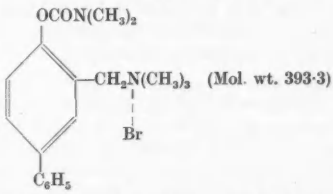
Measurement of enzymatic activity. In all experiments to be reported here the activity of the specific and of the non-specific enzyme was determined manometrically by Warburg's method at 38°. *In vitro*, the activity was measured in a 0·025 M-$NaHCO_3$ solution saturated with 5% Co_2 (pH 7·4), while for experiments *in vivo* the activity was assayed in undiluted plasma, saturated with 5% CO_2. In this latter series a correction for retention (Warburg, 1925) was necessary and all records for enzymatic activity in this section represent corrected values.

Since some of the tissues examined contained a mixture of the specific cholinesterase and the non-specific enzyme, a means of estimating the activity of each enzyme separately was required, if changes in the level of both were to be followed. Such a method was first provided by Mendel *et al.* (1943) when they showed that acetyl-β-methyl choline is hydrolyzed by the specific cholinesterase but not by the non-specific enzyme and that benzoylcholine is hydrolyzed by the non-specific enzyme but not by the specific cholinesterase. Accordingly, acetyl-β-methyl choline (0·03 M) (Mecholyl-Merck) was used in the present investigation to estimate the activity of the specific cholinesterase, and benzoylcholine (0·006 M) to measure the activity of the non-specific enzyme.

In the determinations where benzoylcholine was used as substrate, proof had to be obtained that the hydrolysis of this compound was due entirely to the non-specific

cholinesterase, since Sawyer (1945) has demonstrated that certain tissues, e.g. the liver of some rodents, contain an enzyme, benzoylcholinesterase, which hydrolyzes benzoylcholine but not acetylcholine. It was necessary, therefore, to exclude the possibility that benzoylcholinesterase was present in the blood and tissues examined in this investigation. This could be done by applying a test based on the finding that benzoylcholinesterase, in contrast to the specific cholinesterase, is resistant to 2×10^{-6}M-eserine (Mendel & Gunter, unpublished). Thus, if the activity of an enzyme preparation towards benzoylcholine is completely inhibited by this concentration of eserine, the possibility that benzoylcholinesterase is present would be excluded. In all instances below where benzoylcholine served as substrate this test was employed, and in no case was there evidence of the presence of benzoylcholinesterase.

Properties of Nu-683. The dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Hoffmann-LaRoche Nu-683), which was used as an instrument in this investigation and which will be called Nu-683 in this paper, is a water-soluble, heat-stable, substituted quaternary ammonium salt of the following structure:



(Mol. wt. 393·3)

In contrast to physostigmine, Nu-683 forms a very stable complex with the non-specific cholinesterase. The stability of this complex, which assumed considerable significance when the inhibition of the non-specific enzyme was determined in the *in vivo* series of experiments, is apparent from the following results:

(1) The activity of the non-specific cholinesterase of human plasma is inhibited 95% by 10^{-7}M-Nu-683 and 25% by 10^{-9}M-Nu-683. When, however, human plasma, containing 10^{-7}M-Nu-683 is incubated for 1 hr. at 38° and subsequently diluted 100-fold, the inhibition of the non-specific enzyme does not decrease, as might be expected, in accordance with the diminished concentration of the inhibitor, but remains almost unchanged, an inhibition of 78% being observed.

(2) When dog plasma, containing Nu-683 in a concentration which inhibits the activity of its non-specific cholinesterase 90%, is dialyzed against tap water for 24 hr.—a procedure which does not reduce the activity of the enzyme—the effect of Nu-683 on the non-specific enzyme is only partially abolished, the inhibition still amounting to 77%.

Since the activity of the specific and the non-specific cholinesterases in this investigation was not measured with acetylcholine as substrate but with acetyl-β-methyl choline and benzoylcholine respectively, it was necessary to ascertain whether the inhibition brought about by Nu-683, when these compounds were used as substrates, corresponded to the inhibition observed when the natural substrate, acetylcholine, was employed. Experiments showed that the activity of enzyme preparations containing exclusively either the specific or the non-specific cholinesterase, e.g. dog brain or dog pancreas, when measured in the presence of Nu-683, was inhibited to the same degree no matter whether acetyl-β-methyl choline (0·03M) and benzoylcholine (0·006M) respectively were used as substrates, on the one hand, or acetylcholine (0·0006 or 0·06M), on the other.

EXPERIMENTAL

In vitro *experiments*

Table 1 shows the selective inhibition of the non-specific cholinesterase in human blood and dog plasma by Nu-683. It will be seen that Nu-683 in a concentration of 10^{-8}M inhibits the activity of

Table 1. *Inhibitory action of Nu-683 on the non-specific and the specific cholinesterase* in vitro

Source	Substrate	Conc. of Nu-683 (M)	Inhibition of enzymatic activity (%)
Human plasma	Benzoylcholine	5×10^{-9}	66
Human plasma	Acetyl-β-methyl choline	5×10^{-9}	0
Human erythrocytes	Acetyl-β-methyl choline	5×10^{-9}	4
Human plasma	Benzoylcholine	1×10^{-8}	92
Human plasma	Acetyl-β-methyl choline	1×10^{-8}	0
Human erythrocytes	Acetyl-β-methyl choline	1×10^{-8}	15
Dog plasma	Benzoylcholine	$2{\cdot}5 \times 10^{-8}$	55
Dog plasma	Acetyl-β-methyl choline	$2{\cdot}5 \times 10^{-8}$	0
Dog plasma	Benzoylcholine	5×10^{-8}	91
Dog plasma	Acetyl-β-methyl choline	5×10^{-8}	27

the non-specific cholinesterase of human plasma by 92 %, whereas the activity of the specific cholinesterase of human plasma and erythrocytes is inhibited only slightly, if at all; similarly, $2{\cdot}5 \times 10^{-8}$M-Nu-683 inhibits the non-specific cholinesterase of dog plasma 55 %, without exerting any effect whatever on the activity of the specific cholinesterase.*

* Two factors exclude the possibility of using Nu-683 in routine work to determine quantitatively the activity of the specific cholinesterase and the non-specific enzyme towards acetylcholine when a mixture of both enzymes is present: (1) Nu-683 in a concentration which inhibits almost completely the activity of the non-specific enzyme, always causes a slight inhibition of the activity of the specific cholinesterase; (2) in different species variations exist in the sensitivity of these two enzymes towards Nu-683.

Similar experiments on the selective inhibition of the non-specific enzyme *in vitro* have been done previously. Zeller & Bissegger (1943) showed that 'percain' (American-nupercaine), in a concentration which inhibits only slightly the cholinesterase activity of human brain and erythrocytes, causes almost complete inhibition of the activity of human plasma. On the basis of the findings that brain and erythrocytes contain specific cholinesterase only whereas human plasma contains predominantly the non-specific enzyme (Mendel *et al.* 1943), the results of Zeller & Bissegger can be interpreted as indicating that percain inhibits selectively the non-specific enzyme. Harris & Harris (1944) observed that certain unauthenticated curare preparations inhibited almost completely the activity of the non-specific enzyme in human serum in a concentration which affected only slightly the activity of the specific cholinesterase of human erythrocytes. However, neither percain nor crude curare preparations could be used to inhibit the non-specific enzyme *in vivo* on account of their high toxicity.

In vivo *experiments*

In preliminary experiments, increasing amounts of Nu-683 were injected intravenously into dogs to determine whether this substance could be tolerated. It was found that an injection of Nu-683 which brought the concentration in the animal to about 5×10^{-8} M—assuming a uniform distribution in its tissues—did not provoke the slightest reaction.

Since analysis of blood samples withdrawn 5 min. after such an injection revealed that the activity of the non-specific enzyme had been inhibited about 80 %, it seemed likely that the concentration of the inhibitor could be increased beyond this point to cause a still greater inhibition of the non-specific enzyme, without eliciting any reaction attributable to this inhibition.

Experiments showed that an injection of Nu-683 which brought the concentration in the animal to about $1{\cdot}5 \times 10^{-7}$ M, although inhibiting the activity of the non-specific enzyme 95 %, caused no ill-effects. Table 2 illustrates the degree of inhibition of the non-specific enzyme and of the specific cholinesterase 5, 30 and 60 min. after an intravenous injection of 0·95 mg. of Nu-683 into a dog weighing 15 kg. It will be seen that the activity of the non-specific cholinesterase at first is inhibited 94 %, the inhibition of this enzyme never falling below 80 % during the experimental period; on the other hand, the inhibition of the specific cholinesterase never exceeds 32 %, full activity of this enzyme being observed at a point where only 20 % of the activity of the non-specific cholinesterase remains.

Since the level of inhibition was not maintained in the above experiment and the absence of symptoms under these conditions therefore did not warrant a conclusion regarding the significance of the non-specific cholinesterase, an attempt was made to maintain an almost complete inhibition of this enzyme over a longer period by means of supplementary injections. A continuous infusion method was not employed, since it would have necessitated immobilizing the animal, and freedom of movement seemed preferable in order to allow observation of any symptoms of abnormal behaviour, should they occur. Table 3 gives the results of one of the experiments in which the inhibition of the non-specific cholinesterase was maintained by supplementary injections of Nu-683. Since the degree of inhibition amounts to 89 % at a time when maintenance of the inhibition would have necessitated the administration of a further supplementary injection, it is evident that this figure represents the minimum inhibition prevailing during the experiment. The activity of the specific cholinesterase, on the other hand, was never significantly affected in such experiments, and no reactions, related or unrelated to acetylcholine poisoning, were observed.

In another experiment in which no abnormal reactions were noted, the level of inhibition of the non-specific enzyme in the plasma was maintained above 90 % for 1 hr. 12 min. The animal was killed by an intravenous injection of air 4 min. after the last supplementary dose of Nu-683 had been administered, and the pancreas and superior cervical ganglia were immediately removed and tested for enzymatic activity in order to ascertain whether

Table 2. *Selective inhibition of the non-specific cholinesterase* in vivo

Dog—male, wt. 15 kg.

Time (hr.)	Amount of Nu-683 injected	Activity of non-specific cholinesterase*: CO_2 evolved by 1 ml. plasma in 10 min. (μl.)	Activity of non-specific cholinesterase*: Inhibition (%)	Activity of specific cholinesterase†: CO_2 evolved by 1 ml. plasma in 10 min. (μl.)	Activity of specific cholinesterase†: Inhibition (%)	Remarks
10.00	—	184	—	41	—	—
10.00–10.05	0·95 mg. in 12 ml. saline	—	—	—	—	—
10.10	—	11·4	94	28	32	No symptoms
10.35	—	23	87	34·5	15	No symptoms
11.05	—	36	80	41	0	No symptoms

* Substrate, benzoylcholine (0·006 M). † Substrate, acetyl-β-methyl choline (0·03 M).

Table 3. *Effect* in vivo *of a sustained inhibition of the non-specific cholinesterase*

Dog—male, wt. 26 kg.

Time (hr.)	Amount of Nu-683 injected	Activity of non-specific cholinesterase*: CO_2 evolved by 1 ml. plasma in 10 min. (μl.)	Inhibition (%)	Activity of specific cholinesterase†: CO_2 evolved by 1 ml. plasma in 10 min. (μl.)	Inhibition (%)	Remarks
11.00	—	221	—	50	—	—
11.00–11.05	1·6 mg. in 20 ml. saline	—	—	—	—	—
11.10	—	6·2	97	—	—	No symptoms
11.22–11.28	0·22 mg. in 5·6 ml. saline	—	—	—	—	No symptoms
11.42–11.48	0·22 mg. in 5·6 ml. saline	—	—	—	—	No symptoms
12.02–12.08	0·22 mg. in 5·6 ml. saline	—	—	—	—	No symptoms
12.22	—	24·5	89	38·5	23	No symptoms
18.20	—	83	63	—	—	—

* Substrate, benzoylcholine (0·006 M). † Substrate, acetyl-β-methyl choline (0·03 M).

Nu-683 inhibited not only the activity of the non-specific cholinesterase in the blood stream but the activity of this enzyme in the tissues as well.

Inhibition of the non-specific enzyme in the pancreas and superior cervical ganglia

The pancreas was chosen for investigation because in dogs this organ has been shown to contain very large amounts of the non-specific cholinesterase (Mendel & Mundell, 1943), while the superior cervical ganglia served as an example of nervous tissue which, unlike brain, contains a mixture of the specific and the non-specific enzyme (Mendel & Rudney, 1944).

(*a*) *Pancreas.* The pancreas on removal was finely ground and suspended in nine parts of distilled water. When the activity of this suspension was tested towards acetylcholine (0·02 M) in 0·025 M-$NaHCO_3$, the original tissue having been diluted thirteen times in all, a Q_{Ach}* value of 93 was found.

Since experiments on the stability of the enzyme-inhibitor complex, mentioned earlier in this paper, had demonstrated the difficulty of removing the inhibitor from the enzyme by dilution or dialysis, such procedures were not employed in order to obtain an approximation of the normal Q_{Ach} value for the pancreas under investigation. Instead, a comparison was made with Q_{Ach} values of normal dog pancreas, tested in the same manner as above. The Q_{Ach} values obtained for the pancreas of nine dogs were as follows: 734, 416, 458, 800, 745, 400, 720, 470 and 600; average 594. When the Q_{Ach} value of 93 is compared with these values it is evident that the activity of the non-specific cholinesterase in the pancreas under investigation must have been inhibited at least 77 %, but possibly 88 %, while on the basis of the average normal value, the inhibition amounted to 84 %.

(*b*) *Superior cervical ganglia.* The two superior cervical ganglia, each weighing approximately 20 mg., were finely chopped with scissors, ground and suspended in 0·45 ml. of water.

Preliminary experiments having shown that the Q_{Bch} values for normal dog superior cervical ganglia extend over an extremely wide range, these values could not be used as alternative estimates of the normal content of non-specific enzyme in the ganglia under investigation. An approximation of the normal content of non-specific enzyme in these ganglia could, however, be obtained by diluting the tissue to the greatest possible degree, thereby effecting as complete a dissociation of the enzyme-inhibitor complex as possible, the only limiting factor to the extent of dilution being the presence of sufficient tissue to allow an accurate estimation of enzymatic activity. A 2000-fold dilution fulfilled the above conditions and was used in this estimation, the activity towards benzoylcholine being measured in 0·025 M-$NaHCO_3$.

In the second estimation of the activity of the non-specific enzyme in the ganglia, dilution of the ground tissue was kept to a minimum, in order to avoid as far as possible a dissociation of the enzyme-inhibitor complex. The minimum imposed by the experimental conditions in this case was a 40-fold dilution.

A comparison of the results of these two estimations—on the basis of equal amounts of enzyme—revealed that the activity of the 40-times diluted sample was only 32 % of that of the sample which had been diluted 2000 times in all. This calculation, however, is open to two sources of error, both of which would prevent an appreciation of the full extent of the inhibition actually prevailing in the ganglion: first, a 40-fold dilution will cause some slight dissociation of the enzyme-inhibitor complex, secondly, even a 2000-fold dilution probably will

* The symbol Q is used here to designate the activity of an enzyme preparation, expressed as μl. CO_2 evolved by 1 mg. (dry wt.) of this preparation in 1 hr. The suffix to Q indicates the substrate used, i.e. Ach = Acetylcholine and Bch = Benzoylcholine.

not cause complete dissociation of the complex. An inhibition of 68 %, observed under these conditions, therefore, can be considered an approximation only, the actual inhibition, in all probability, being much greater.

In summary it can be said that Nu-683 not only inhibits the activity of the non-specific cholinesterase in the blood stream, but passes from the circulatory system into the tissues, and inhibits the activity of the enzyme at this site as well. No symptoms related to acetylcholine poisoning appeared, however, in spite of the almost complete inhibition of the activity of the non-specific enzyme in blood and tissues.

In the experiments reported so far, the activity of the non-specific cholinesterase in blood and tissues was almost completely inhibited, while the activity of the specific cholinesterase was only slightly depressed. Further experiments were carried out to investigate the reactions of animals in which, by the injection of a larger amount of Nu-683, not only the activity of the non-specific enzyme was almost completely inhibited, but the activity of the specific cholinesterase was significantly reduced as well. Table 4 illustrates one set of experimental results obtained under these conditions. It is evident that the reactions elicited in the animal, the most striking of which were severe intestinal disturbances, excessive salivation and generalized fibrillation of the surface musculature, are symptoms characteristic of acetylcholine poisoning. These symptoms appeared at a time when the inhibition of the activity of the specific cholinesterase in the plasma amounted to 77%.

When a similar dose of Nu-683 was injected one week later into the same animal after the administration of 4·3 mg. of atrophine sulphate subcutaneously, all the symptoms displayed previously, with the exception of fibrillation, were absent (fibrillation caused by acetylcholine would obviously not be abolished by atropine). These results indicate that the effects observed when Nu-683 was injected into an unatropinized animal must have been due to acetylcholine and not to side reactions induced by the increased amount of Nu-683.

It is evident, therefore, that symptoms characteristic of acetylcholine poisoning fail to appear when an almost complete inhibition of the activity of the non-specific cholinesterase has been brought about and that these symptoms are not elicited unless a concomitant inhibition of the activity of the specific cholinesterase has been effected.

DISCUSSION

Our experiments have demonstrated that an almost complete inhibition of the non-specific cholinesterase in an animal does not evoke any reaction indicative of the accumulation of acetylcholine. Thus, it is apparent that the non-specific cholinesterase, although capable of hydrolyzing acetylcholine *in vitro*, plays no essential part in this process *in vivo*. The term pseudo-cholinesterase, pointing to the deceptive similarity between the non-specific enzyme and the specific cholinesterase, therefore seems indeed appropriate.

In the light of present knowledge, the role of pseudo-cholinesterase in the process of nerve impulse transmission could be at best a minor one, that is, in serving as a comparatively ineffective substitute for the specific or true cholinesterase in conditions in which the activity of the latter enzyme is depressed or impaired. Proof of this hypothesis, however, remains to be established.

Table 4. *Effect* in vivo *of a significant inhibition of the specific cholinesterase in addition to the inhibition of the non-specific enzyme*

Dog—male, wt. 11·4 kg.

Time (hr.)	Amount of Nu-683 injected	Activity of non-specific cholinesterase*: CO_2 evolved by 1 ml. plasma in 10 min. (μl.)	Activity of non-specific cholinesterase*: Inhibition (%)	Activity of specific cholinesterase†: CO_2 evolved by 1 ml. plasma in 10 min. (μl.)	Activity of specific cholinesterase†: Inhibition (%)	Remarks
2.10	—	280	—	42	—	—
2.15–2.18	5·0 mg. in 12 ml. saline	—	—	—	—	—
2.20	—	—	—	—	—	Defecation, tremor of hind limbs
2.21	—	—	—	—	—	Diarrhoea
2.24	—	—	—	—	—	Yawning, dyspnoea, excessive salivation, fibrillation
2.25	—	4·1	98·5	9·6	77	Extensive diarrhoea, coughing
2.30	—	—	—	—	—	Diarrhoea, salivation and fibrillation continuing
2.35	—	—	—	—	—	Vomiting

* Substrate, benzoylcholine (0·006 M). † Substrate, acetyl-β-methyl choline (0·03 M).

SUMMARY

1. It has been found that a prostigmin analogue, the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Hoffmann-LaRoche Nu-683) is capable of inhibiting selectively the activity of the non-specific or pseudo-cholinesterase.

2. Injections of this compound have demonstrated that an almost complete inhibition of pseudo-cholinesterase in the blood and tissues of dogs can be effected without eliciting symptoms indicative of the accumulation of acetylcholine.

3. Symptoms indicative of the accumulation of acetylcholine appear only when the activity of the specific or true cholinesterase is significantly depressed as well.

4. These results show that pseudo-cholinesterase plays no essential part in the hydrolysis of acetylcholine *in vivo*.

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The Extraction of Nitrogenous Materials from Green Leaves

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Our knowledge of plant proteins has been reviewed by Vickery (1945). This shows how meagre is the information on the distribution of proteins in green leaves compared with that on proteins in seeds and grains. This difference can partly be explained by the dietetic value of the latter, but it is also a reflexion of the difficulty attending the extraction of the nitrogenous compounds from leaves in forms in which they can be handled conveniently. A systematic study of grinding methods and extracting reagents has therefore been made, to find a short, simple and efficient process for dispersing the nitrogenous compounds of the leaf.

Rouelle (1773) first showed that protein occurs in parts of the plant other than the seed, and by heat coagulation separated the juice of hemlock leaves into a green part and one almost free from pigment. The aim of most subsequent work has been the analysis of the product rather than the preparation of the proteins in a form in which their physico-chemical properties are as little changed as possible. In consequence, many of the extraction procedures were rather drastic, e.g. the use of 60 % ethanol containing 0·3 % NaOH (Osborne, 1924).

Modern work on the extraction of leaf proteins begins with Osborne & Wakeman (1920) who used spinach and Chibnall & Schryver (1921) who used cabbage. By grinding with water or ether-water they obtained about 60 % of the leaf nitrogen in solution. By grinding runner-bean leaves in an end-runner mill with six separate lots of water, Chibnall (1922) was able to separate 80–90 % of the total leaf N from the rest of the cell debris. Noack (1927) and Menke (1938) also separated a portion of the N by simple grinding with water. The 'protein' prepared by heat coagulation of such extracts contains only 11–12 % N and much ash (10–12 %). In an endeavour to obtain purer preparations, Chibnall (1923, 1924) and his co-workers (Chibnall & Grover, 1926; Miller & Chibnall, 1932; Chibnall, Miller, Hall & Westall, 1933) elaborated the well-known method of cytolyzing the leaf with ether or ether-water, pressing out the 'vacuolar' fluid containing a small amount of 'vacuolar' protein and dispersing the remaining 'cytoplasmic' protein by grinding and water extraction. By acid treatment of the extracts they obtained products containing 16·25 % N on an ash-free basis, but in yields of only 40 % or less of the nitrogen of the leaf.

The highest extractions reported to date are those of Lugg (1939). By fine grinding in an end-runner mill in the presence of a large volume of borate buffer, pH 9·2, followed by gentle centrifugation to separate the cell debris, he claims extractions on a small scale of 92–95 % of the total leaf N.

EXPERIMENTAL

Leaves. Tobacco (*Nicotiana tabacum* var. White Burley) has been used because of its availability throughout the year and because of its importance in virus studies. Experiments have also been done with *N. glutinosa*, *Phaseolus vulgaris* var. Canadian Wonder and with *Datura stramonium*. The plants were grown in glasshouses with heat but no extra illumination. The potting compost contained small amounts of added N, P and K and a few batches received top dressings of $(NH_4)_2SO_4$. As far as possible plants about 6 in. high were used. Senescent and very young leaves were discarded. The leaves were cut immediately before beginning extractions, the delay always being less than 20 min. Variations in dry matter and nitrogen content occurred with the size and age of the plants, weather conditions, manurial treatment and the time of day and the season at which they were picked. Although they affect the extraction of nitrogen to some degree, no attempt has been made to control or allow for these variations. In the tables, average figures for extraction are given except in three instances (Tables 3, 10 and 14).

Cutting and grinding machinery. The machines used were a domestic meat mincer, a Latapie mincer, a commercial food cutter, a triple-roller ointment mill, an end-runner mill, a Torrance 'N.T.' conical edge-runner mill and a power-driven laboratory agate mortar. The triple-roller mill has already been described by Bawden & Pirie (1944) and the end-runner is of the conventional laboratory pattern. The food cutter is made by the Hobart Manufacturing Co. of U.S.A. and consists of a rotating bowl carrying the leaves under a set of sharp knives rotating at 1450 r.p.m. The cutting is done with a slicing action and a minimum of incidental cutting and bruising. The N.T. mill is made by Torrance and Sons Ltd., of Bristol, and consists of a cast iron conical bowl of 13 in. diameter, of semiapical angle approx. 60°, rotating under a cast iron conical edge runner with the wide end orientated towards the centre. The inner and outer edges of the runner therefore move considerably faster and slower respectively than the corresponding portions of the bowl. A shearing action is thus introduced into the grinding. A high-speed shaking machine running at 225 strokes/min. was also available. In this small quantities of leaf pulp suspensions could be shaken with sand according to the technique of Curran & Evans (1942).

Analyses. Nitrogen was determined by a micro-Kjeldahl procedure using $1:1:8$ $SeO_2:CuSO_4:K_2SO_4$ catalyst. Markham's (1942) still was used, ammonia being collected in 2% boric acid and titrated directly with N/28-HCl. Dry matter was obtained after drying overnight in an electric oven at 95–100°. To obtain an approximation to the amount of protein in extracts, precipitation by 4% trichloroacetic acid has been used. Included in the precipitate will be porphyrin nitrogen and that of N-containing lipids, both of which are included in the plastid fragments which precipitate under these conditions, as well as possible unknown N-containing compounds. For this reason it has seemed desirable not to prejudice the issue by referring to the extraction of 'protein' solely on the basis of total and trichloroacetic-precipitable N estimations, but to leave the matter open for the present and refer only to the extraction of *nitrogen*.

General notes on procedure. Distilled water was used throughout. As the aim has been to prepare the proteins of the leaf with a minimum amount of alteration, all extraction procedures were as mild as possible. Thus, cytolysis with ether or ether-water was provisionally ruled out as being too drastic, because Chibnall and his co-workers had often found difficulty in dispersing the 'cytoplasmic' material after such treatment. The pH of extracting fluids was never allowed to exceed 8·0 since many proteins are altered above this value, e.g. potato virus *Y* and Hyoscyamus virus 3 lose their infectivity at pH 9 (Bawden & Pirie 1939) and many enzymes, e.g. trypsin, are inactivated rapidly above pH 8 (Northrop, 1939).

Freshly minced leaves were squeezed without dilution. For making extracts from fibres from which sap had already been removed, the leaf material was suspended in such a volume of extraction fluid that the final dry-matter content of the mixture was between 5 and 10%.

Squeezing was done by hand through a fine cotton cloth (madapollam), the residue washed twice or thrice with a volume of water equal to one-half that of the extracting fluid (except where noted) and the combined extracts centrifuged for 15 min. at approximately 1500 × gravity. In the earlier experiments, the precipitate thus obtained was returned to the residue for analysis or further extraction.

RESULTS

Grinding methods

Preliminary grinding. In comparing the efficiency of the various methods of grinding, no attempt was made to control the pH. Extractions were done with distilled water and hence at the natural pH of the leaf.

The mincer and food cutter being best suited to the preliminary reduction of large amounts of fresh leaves, comparisons of their efficiency at this stage were made. Table 1 shows the extractions thus obtained from tobacco and beans. Mincing is seen to extract more nitrogen from both species, the nitrogen/dry-matter ratio being in favour of the mincer with tobacco and in favour of the cutter with beans. The additional efficiency of the mincer is shown by mincing the residue obtained after separating the juice from the leaves cut in the food cutter. With tobacco, the extra extraction thus obtained amounts to 12 % of the leaf nitrogen and 10 % of the leaf dry matter, i.e. extractions with mincer alone are very similar to those with cutter + mincer. The mincer has the additional advantage of being able to handle small as well as large quantities of leaves and has therefore been used in almost all experiments as a preliminary cutter. A second mincing brings out a small extra amount of juice, after which the material is washed as above.

Table 1. *Comparative efficiency of food cutter and mincer for preliminary grinding of leaves*

	% extraction of			
	Soluble N		Soluble dry matter	
Species	Food cutter	Mincer	Food cutter	Mincer
Tobacco (*Nicotiana tabacum*)	35	48	35	45
Bean (*Phaseolus vulgaris*)	37	42	31	37

Following Bawden & Pirie (1944), the material obtained after mincing and washing will be referred to as 'fibre'. It consists mainly of leaf fragments in which the cells, although apparently whole when examined microscopically, have been so damaged as to upset their osmotic equilibrium. Precipitates obtained after centrifuging the juice of the leaves plus washings or from any other extracts will be called, for convenience, 'chloroplasts'. They consist mainly of fine cell debris, plastids and plastid fragments and varying amounts of starch. The solution from the centrifugation of leaf juice plus washings will be called 'sap'. Any material remaining after extractions involving operations other than mincing and washing will be referred to as 'fibre residue' or simply 'residue'.

50 % of the nitrogen in the sap of tobacco is, on the average, precipitable by trichloroacetic acid. There are considerable variations around this average depending on the state and age of the leaf, the season, manuring, etc. The extreme values found were 30 and 68 %, but two-thirds of the results fell within the 40–60 % range. The average extractions of nitrogen and dry matter by mincing and washing are 45 and 43½ % respectively of the amounts of these constituents in the intact leaf.

Fine grinding. For finer grinding a comparison was made of the efficiencies of the Latapie mincer, triple-roller mill, Torrance N.T. mill, end-runner mill and the agate mortar. The latter was found wholly unsuitable; the particles of fibre bunch together at the corners of the pestle, which is of the large rectangular type, and are not carried under and crushed. The edge- and end-runner mills had the apparent advantage that the material could be more easily subjected to a larger number of grinding cycles than is possible with the roller mill. Each machine requires fibre of a certain minimum water content before grinding is satisfactory. The Latapie and roller mill will handle fibre as it is obtained after squeezing, i.e. containing 20–25 % dry matter. It is necessary to add an equal volume of water before grinding in the edge-runner and no less than 8 vol. in the end-runner. With the latter, the residue was washed only once. For comparative experiments fibre was put through the Latapie once, the roller mill twice, and ground in the edge- and end-runners for 5 min. These were the treatments after which no further perceptible change occurred.

Table 2. *Comparative efficiency of machines for fine grinding of tobacco fibre*

	% of component in fibre extracted			
	Nitrogen		Dry matter	
Mill	Soluble	'Chloroplastic'	Soluble	'Chloroplastic'
T.R.M.	15	42	8	31
N.T.	11½	30	8	24½
E.R.	10	19	4½	16½
Latapie	14	—	9½	—

T.R.M., Triple-roller mill; N.T., Torrance, N.T. conical edge-runner mill; E.R., End-runner mill.

It can be seen from Table 2 that under these conditions the roller mill was more efficient than any of the others. 'Chloroplast' figures are not given for the Latapie because this machine so chops up the fibre that considerable amounts of cell debris come through the cloth and are included with this fraction. For this reason, and because its use is so laborious for weights of fibre above 10 g., the Latapie was not investigated further. Not only are the extractions with the edge- and end-runners less, but they have the added disadvantage of requiring more time to grind and clean, and of contaminating the extracts with iron.

The triple-roller mill. This was investigated further to find the optimum conditions of grinding (cf. Bawden & Pirie, 1944). Washed fibre was pressed in a hydraulic press to 4000 lb./sq.in., thereby raising the dry-matter content to about 55 % in a typical experiment. Portions of this fibre were mixed with water to give the dry-matter contents shown in Table 3 and each was passed through the mill twice,

Table 3. *Extractions after milling fibre at different water contents*

	% of component in fibre extracted					
	Nitrogen			Dry matter		
Dry matter content (%)	Soluble	'Chloroplastic'	Total	Soluble	'Chloroplastic'	Total
53·1	17	26	43	21	15½	36½
31·9	13	33	46	15½	23½	39
25·8	15	31	46	11½	28½	40
21·2	13	36	49	12½	29	41½
10·6	13	27	40	9	26½	35½
5·3	11	25	36	7½	25½	33

and extracted with water in the standard manner. Preliminary experiments showed that the fibre reaches equilibrium with the added water almost immediately and that pressing has little or no effect

on the degree of extraction. Thus the variations in extraction shown in Table 3 are due to the different moisture contents during milling.

There is a pronounced decrease in the amount of soluble dry matter extracted with decrease in dry-matter content of the fibre milled. When plotted, these figures fall almost on a straight line. The trend in soluble nitrogen is not nearly so pronounced, although, on the whole, it is in the same direction. The effect of the faster falling off in dry-matter extraction is that extracts from wetter fibre are richer in nitrogen. In the chloroplastic fractions, both nitrogen and dry matter show a maximum in the neighbourhood of 20 % dry-matter content, rather more pronounced in the case of dry matter. Throughout the range, the ratio of nitrogen/dry matter in this fraction increases with increasing dry-matter content during milling.

The extractions of total dry matter and total nitrogen show maxima at the same point as the chloroplastic fraction, viz. 20 % dry-matter content. The quantitative picture coincides well with that obtained from experience of the milling process, for, judging from the 'feel', the most satisfactory grinding occurs when the dry-matter content of the fibre is between 15 and 25 %.

Extraction methods

Washing. Serial washing of fibre and milled fibre was investigated to find the most useful degree of washing. Chibnall and his co-workers had used water saturated with ether as a cytolytic agent, and Lugg (1939) showed that the addition of ether to the extracting fluid favours the extraction of nitrogen from the chloroplastic residues. Saturated ether-water was therefore compared with distilled water as a wash fluid.

Table 4. *Effect of serial washing with water and ether-water on the extraction of nitrogen and dry matter from minced and from milled fibre*

	% of original leaf component extracted			
	Soluble nitrogen		Soluble dry matter	
Treatment	Water	Ether-water	Water	Ether-water
Mince and squeeze	39	39	28	28
1st wash	5	4½	4	5½
2nd wash	1½	1	1	1
3rd wash	1½	1	½	½
4th wash	1	½	½	½
5th wash	1	½	½	½
Milled				
6th wash	5	4	3	2½
7th wash	4	3½	2	1½
8th wash	1½	1	1	1
Total extraction	59½	55	40½	41

Portions of unwashed fibre were washed five times with fluid in the proportion of 100 ml. to 50 g. of fibre. They were then milled and the washings repeated three times more. The results are shown in Table 4. Ether-water is seen to be less efficient than distilled water; in every wash it extracts less N, whilst extracting as much or a little more dry matter. The residual fibre from the water washings contains 1·7 % N and that from the ether-water wash 1·9 %. It will be seen that three washes are all that are useful at each stage and this has been made the standard procedure.

Freezing. This is a well-known method of setting free cell contents. Table 5, however, shows that it decreases the extraction from fibre. The decrease is

Table 5. *Effect of freezing on extraction of fibre*

	% of component in fibre extracted			
	Soluble nitrogen		Soluble dry matter	
Species	Frozen	Not frozen	Frozen	Not frozen
Bean	8½	10½	10	9½
Tobacco	12	17½	8½	10½

greater for tobacco than for beans and the extraction of nitrogen is more seriously affected than the extraction of dry matter. The effect of freezing is more pronounced than that shown in the table if the whole mince is frozen before squeezing and washing. Freezing the mince denatures protein, which would otherwise pass into the sap, and retains it in the fibre. This denaturation is probably the most important cause of the reduced extractions, and in fact virus workers have used freezing as a means of clarifying the deep green extracts from minced and milled leaves. The present experiments strongly support the statement of Bawden & Pirie (1938) that, '...freezing the tissues may be a dangerous preliminary to work on the labile protein constituents of plants'.

Alkaline extraction. As the isoelectric point of most of the leaf proteins appears to be in the neighbourhood of pH 5, it is to be expected that mild alkaline treatment should increase their solubility. Further, as cellulose and other polysaccharides imbibe more water and become softer as the pH is raised, an increase should also assist extraction by increasing the ease of milling. The upper limit of pH is set only by the susceptibility of the proteins to injury and has been fixed at pH 8·0 for reasons already stated.

Washed fibre was suspended in 6–7 times its own weight of water to give a mixture of convenient consistency. In raising the pH of such a mixture to 8, the acid drift described by Holden (1945) was encountered. However, after 6–7 hr., this had become so slow that the mixture could be squeezed out and the fibre milled without any appreciable

decrease in pH. In this time the alkali taken up was 18–20 ml. of 0·2N-NaOH/100 g. fibre. After milling, the material was again suspended in 6–7 times its weight of distilled water and again adjusted to pH 8·0. Usually only 2–5 ml. of 0·2N-NaOH/100 g. milled fibre were required and very little drift occurred. Squeezing and washing were then carried out as before.

Table 6 shows the increase in extraction obtained by alkali treatment. On the left are the average

Table 6. *Effect of dilute* NaOH *on extraction of tobacco fibre by simple washing and by grinding*

	% of component in material extracted			
	Soluble nitrogen		Soluble dry matter	
Treatment	Washing	Milling after washing	Washing	Milling after washing
Water extract (pH 6·4)	5	21	3	12
NaOH extract (pH 8·0)	18	66	9	24½

extractions with distilled water under the same conditions as with dilute NaOH (below). The average extraction of nitrogen is raised by a factor of 3½ and that of dry matter by a factor of 3. On the right are the extractions from milled fibre expressed as a percentage of the nitrogen or dry matter remaining after washing. Again, the alkali treatment has raised the extraction of nitrogen by a factor of 3 and of dry matter by a factor of 2, and this in spite of the fact that the alkali-washed fibre has already had three times as much nitrogen and dry matter removed. An added advantage is that the nitrogen/dry matter ratio is increased, i.e. proteins are being preferentially extracted.

Since the average extractions of nitrogen and dry matter by mincing and washing are 45 and 43½ %, the average total extractions resulting from these additional processes amount to 58½ and 51½ % nitrogen and dry matter at the natural pH of the leaf fibre, and 85 and 61 % nitrogen and dry matter respectively by extracting at pH 8·0.

The amount of trichloroacetic acid precipitable nitrogen is higher in these extracts than in sap. On an average, 64 % of the nitrogen in the neutral extracts precipitates, the extremes being 41 and 81 %. The precipitable nitrogen in the extracts after milling is 91 % (average), ranging between 88 and 94 %. Thus there is a progressive washing out of the non-precipitable nitrogenous materials in the earlier stages, but this is not complete before the fibre is milled. As the average extractions at the mincing, neutralization and milling stages are approximately 45, 10 and 30 % of the total leaf nitrogen of tobacco, the amounts of 'protein' nitrogen are 23, 6 and 27 % of the total nitrogen. Assuming that the 15 % of the total leaf nitrogen remaining in the milled fibre is 'protein-N', a total of 71 % of the leaf nitrogen is in the form of 'protein'. The average nitrogen content of the leaves used in these experiments was 3·5 %. The 'protein-N' content was therefore 2·5 % which corresponds to a 'protein' content of 15 % of the dry matter. Of this, 85 % is extracted.

Experiments involving extractions of residues from the above treatments at pH 10·2 with dilute NaOH or pH 9·2 with ice-cold 10 % ethanol (cf. Lugg, 1939) are summarized in Table 7. It can be seen that further extractions, even under these rather drastic conditions, result in quite small extra yields, while they probably damage the proteins considerably.

Table 7. *Extraction of alkali* (pH 8·0)*-treated residues at higher alkalinities*

	% of residual component in treated fibre extracted	
Treatment	Soluble nitrogen	Soluble dry matter
NaOH (pH 10·2)	12	3½
10 % ethanol (pH 9·2 at 0°)	21	4½

Alkaline earth metal hydroxides offer a convenient means of maintaining a high pH. $Mg(OH)_2$ was chosen for experiment, in spite of its equilibrium pH being 9·7, i.e. above the value considered 'safe' in these experiments. Suspensions containing 1 % of $Mg(OH)_2$ were used both for soaking fibre to pH equilibrium and as extracting fluid after milling. No drift was detectable in $Mg(OH)_2$-treated samples but, as can be seen from Table 8, extractions were low and the decrease in nitrogen extraction greater than the decrease in dry-matter extraction. This effect is presumably due to the Mg^{++} ion.

Table 8. *Extraction of washed fibre by* $Mg(OH)_2$ *suspensions* (pH 9·6) *compared with* NaOH (pH 8·0) *solutions*

	% of component in fibre extracted			
	Soluble nitrogen		Soluble dry matter	
Reagent	Washing	Milling after washing	Washing	Milling after washing
NaOH (pH 8·0)	19½	67	10½	26
$Mg(OH)_2$ (pH 9·6)	5	17	6½	13½

Buffers. The chief drawback to the use of NaOH is the time consumed in bringing the fibre sufficiently close to pH equilibrium to ensure the milling being done at pH 8·0. Suitable buffers in this range are few. As nitrogen is to be estimated in the extracts,

the use of nitrogenous buffers, which are the chief members of the mildly alkaline class, was excluded. Phosphate, the most obvious choice, was, on trial, found to cause the precipitation of large amounts of calcium phosphate which entrained nitrogen. The precipitate contained up to 4 % nitrogen on a dry-weight basis. Both borate and carbonate buffers were tried, although pH 8·0 falls at the end of the borate range and between the two pK's of carbonic acid, where the buffering is least. The only substance found, which was sufficiently inert chemically to be used in protein solutions and which had a pK in the correct range, was *o*-chlorophenyl (pK 8·4). An M/15 solution has sufficient buffering power to hold the pH of washed fibre constant up to 24 hr. with the standard ratio of fibre to volume of solution.

The buffering power of carbonate and borate buffers at this pH is too small to prevent serious drift, but it reduces the extent. Thus, in the time necessary for NaOH adjusted fibre to drift back to pH 5·8, carbonate extracted fibre had reached only pH 7·5. Equilibrium is thus established more quickly with the buffers and from this point of view they have an advantage. The drift was corrected by adding the more alkaline component of the buffer and final concentrations when the mixture was squeezed and milled are given in Table 9.

It can be seen from this table that buffers extract nitrogen and dry matter to the same or a smaller extent than NaOH alone. Even when the pH is allowed to rise to 8·6 by the use of sodium borate alone this picture is not altered—little extra extraction results. Even with chlorophenol, where the pH remained constant, yields were considerably depressed.

Salt solutions. That this deleterious effect of buffers is probably explained by the increase in salt concentration follows from experiments with sodium chloride solutions as extractant. The results of a typical experiment are shown in Table 10. Even so small a concentration as M/1000 reduces the amount of soluble nitrogen. The effect of small concentrations is more pronounced when the fibre is not neutralized, although at higher concentrations the percentage decrease is approximately the same (25 %) whether neutralized or not.

Although the reduction of soluble nitrogen is evident from the lowest concentration, the total nitrogen (soluble + chloroplastic) extracted remains approximately constant until the NaCl concentration reaches 0·1 M. Either the chloroplast material is not being divided so finely in the presence of salt, or that already dispersed is being partly flocculated. The extraction of dry matter is affected in the same way. The apparent increase in the amount of material extracted with M-NaCl may be due to the inaccuracy of estimating the distribution between the extracts and the fibre residue of the considerable amounts of sodium chloride involved.

Grinding in buffer. Lugg (1939) had obtained good extractions by grinding leaves without previous mincing in an end-runner mill with large volumes of M/40 Na borate of pH 9·2. His technique has been tried for comparison with the standard mincing and

Table 9. *Comparative efficiencies of buffer solutions and* NaOH *for extraction of fibre*

		% of component in material extracted							
		Soluble nitrogen				Soluble dry matter			
		Washing only		Milling after washing		Washing only		Milling after washing	
Buffer	Final conc. of buffer (M)	Buffer	NaOH	Buffer	NaOH	Buffer	NaOH	Buffer	NaOH
Na_2CO_3-$NaHCO_3$	0·014	14	14	76	76	8½	7	27	23
Na_2CO_3-$NaHCO_3$	0·53	24	24	32	67	15½	11	22	28
Borate	0·022	17½	22	54	51	6½	5	23	23
o-Chlorophenol	0·065	11½	16	37	58	11½	13½	23	23½

Table 10. *Extraction from washed and milled fibre by dilute sodium chloride solutions*

	% of component in washed fibre extracted							
	Nitrogen				Dry matter			
	Not neutralized		Neutralized		Not neutralized		Neutralized	
NaCl (M)	Soluble	'Chloro-plastic'	Soluble	'Chloro-plastic'	Soluble	'Chloro-plastic'	Soluble	'Chloro-plastic'
0·000	12	17	24	24	8	13	14½	13½
0·001	9	18½	21	27½	7	10½	13	16
0·01	—	—	20	29	—	—	12	16½
0·1	9	17	17	24	5½	11	12½	13½
1·0	—	—	17	19½	—	—	20½	15

milling in the triple-roller mill at pH 8·0. For extractions at pH 8·0, the chlorophenol buffer described above was used, at a strength of M/20 (ionic strength equivalent to Lugg's borate). To ensure that differences would not be attributable to the difference in buffers, experiments were also done in the end-runner mill with M/20 chlorophenol buffer at pH 9·2. According to Lugg, any attempt to separate the extract from cell debris by squeezing through cloth, as has been done in the present experiments, results in lowered yields because of retention of chloroplastic material by the remainder of the cell debris. Centrifugation and squeezing were therefore compared in all three buffers.

Batches of 8 g. of tobacco leaves were ground for 5 min. in the end-runner mill with 50 ml. buffer, and washed out with the minimum amount of distilled water. One of each of the buffer extracts was squeezed through cloth and the residue washed three times with 15–20 ml. of water, and one of each was separated according to Lugg with the following modifications. It was found impossible to separate the debris of these leaves by 2 min. centrifugation at 100 × gravity; much material remained suspended and the precipitate was not sufficiently compact for the supernatant fluid to be poured away. It was found necessary to increase the field to 500 × gravity, and this caused the separation of some chloroplastic material. As much as possible of this was removed by washing twice with a volume of water equal to four or five times the volume of the precipitate. All extracts were centrifuged 15 min. at 1500 × gravity to separate chloroplastic material. The results are set out in Table 11.

extract contained a substantial layer of fine cell debris. If the initial separation had been done in lower fields this debris would presumably have been present in larger amounts. The presence of this extra cell debris may account in part for the higher apparent extractions observed by Lugg, although it is possible that the grasses used by Lugg, by reason of their more fibrous leaves, separate more easily at these low fields. The amount of fine debris is a little greater in extracts squeezed through cloth, as might be expected, but the whole chloroplast fraction is greater when prepared thus. It is noticeable, too, that the chloroplastic fraction is greater in extracts at the lower pH. Presumably, one effect of a higher pH is to make more of this fraction soluble. Half the increase in soluble nitrogen between pH 8·0 and 9·2, using chlorophenol buffers, could be accounted for by decrease in the chloroplastic fraction.

Even at pH 9·2, the extractions of soluble material are not as good as those achieved by the mincing, neutralizing and milling at pH 8·0 (80 % at best instead of 85 %). An added disadvantage of the technique is that relatively small amounts of leaves, not more than 50 g., can be handled with apparatus of normal capacity.

Detergents and Ca-*reagents.* As detergents disperse proteins, a mild detergent might be expected to assist extraction of proteins from fibre. Calgon (sodium hexametaphosphate) was chosen as a suitable mild detergent since it has the added advantage of forming non-ionized Ca complexes (Hatch & Rice, 1939), hence preventing the considerable amounts of leaf calcium from forming any insoluble Ca-protein complex. To investigate the importance

Table 11. *Extraction of nitrogen and dry matter by grinding whole tobacco leaves in an end-runner mill in presence of large volumes of buffers*

			% of N and dry matter in leaf extracted			
			Nitrogen		Dry matter	
Method	Buffer	pH	Soluble	'Chloroplastic'	Soluble	'Chloroplastic'
Grind, separate debris by low speed centrifugation	Borate	9·2	73	10	49	10
	Chlorophenol	9·2	76	6½	49	10
	Chlorophenol	8·0	61	11½	54	9
Separate debris by squeezing in cloth	Borate	9·2	77	14	50	30
	Chlorophenol	9·2	80½	8½	47	24
	Chlorophenol	8·0	60	20	54	20

Borate buffer M/40. Chlorophenol buffers M/20.

Lugg's extractions were calculated on the basis of 'soluble' + 'chloroplastic' nitrogen. On this basis, the extractions with tobacco leaves did not reach the values observed by Lugg with certain representatives of the *Graminae*. Even when the separation of cell debris has been done in a centrifugal field five times that used by Lugg, the chloroplastic precipitate from the 1500 × gravity centrifugation of the

of the latter action, experiments were also done with oxalate and citrate solutions as extracting fluids. Oxalate removes Ca in the form of a crystalline precipitate having minimal absorptive powers, and citrate forms non-ionized complexes similar to those with calgon.

All extractions were done at pH 8·0, but the presence of calgon necessitated a different washing

procedure. Its presence made milled fibre slimy and greatly increased its water-holding power. It was impossible to squeeze this in a cloth because the slimy material came through the pores. It was thus necessary to wash by centrifugation, which resulted in less thorough washing. To obtain comparable results, the control experiments with NaOH and experiments with oxalate and citrate were performed in the same way.

Table 12. *Extraction at* pH 8·0 *of* N *and dry matter from washed fibre after soaking and milling in the presence of various reagents*

	% N and dry matter in material extracted			
	Nitrogen		Dry matter	
Reagent	Soak	Mill	Soak	Mill
NaOH	17½	41	10	16
1% 'calgon'	24	53	16	16
1% oxalate	15	49½	8	17½
1% citrate	22	26½	10½	14½

Table 12 sets out the average figures. Calgon increases the extraction of nitrogen both during the adjustment of pH and after milling. At the neutralization stage calgon causes a larger percentage increase in dry matter than in nitrogen extraction, but after milling the opposite is true, i.e. material richer in nitrogen is extracted by calgon after milling but not before. Oxalate depresses the yield of nitrogen at the soaking stage and increases it somewhat after milling, although the total increase is not sufficient to make much difference to the over-all extraction. Citrate has an opposite effect, the reduction of extraction after milling being considerable.

These results indicate that the detergent action of calgon is more important than its Ca-masking action. Calgon is known to be an extremely effective 'anti-Ca' reagent; e.g. Hatch & Rice (1939) found that 1 or 2 parts/million of calgon would prevent the precipitation of 500 parts/million of $CaCO_3$. However, the total amounts of both oxalate and citrate used were two or three times the amount equivalent to the whole leaf Ca, a large part of which is not in solution and probably not involved in this effect.

In spite of its useful nitrogen-extracting powers, calgon has not been generally used in further experiments. As well as making the material slimy and difficult to handle it is difficult to remove.

Extraction sequences

The extractions thus far described are based on the standard operations: mince, wash, soak with dilute reagent at pH 8·0, mill, wash. In most experiments, too, the chloroplastic fraction has been mixed with the remaining fibre to be re-treated in the next extraction. There are several possible variants of this sequence, e.g. the chloroplasts may be regarded at each stage as a separate fraction and not remixed; neutralization of the fibre may be done after milling instead of before; it is possible to carry out more than one milling and washing of the fibre; it would be possible to treat the fibre further in an endeavour to extract residual nitrogen.

Separation of chloroplasts. Although the simplest criterion of extraction was the amount of 'soluble' nitrogen (defined here as material not sedimenting in 15 min. in a field of 1500 × gravity) and dry matter it became desirable at this stage to determine how much of the nitrogen could be separated from the fibre even if it was not all soluble. The observation of Bawden & Pirie (1944) that milling of purified bushy stunt virus with fibre from healthy leaves attaches the virus to chromoproteins made it possible that extraction was being reduced by grinding fibre and previously separated chloroplasts together.

To investigate whether this was the case, chloroplasts were milled with fibre paper pulp. The pulp was prepared by disintegrating filter paper in warm 10 % (w/v) NaOH to hydrate and soften the cellulose so that it would mill more easily. It was then thoroughly washed with water. The damp pulp was mixed with approximately its own weight of chloroplast paste, milled, suspended in about twice its weight of water, and the pH adjusted to 8·0. Controls were done on the unmilled mixture; squeezing, washing and centrifugation was carried out as usual.

Table 13. *Effect of milling chloroplasts with filter paper pulp*

	% of component in mixture extracted			
	Nitrogen		Dry matter	
Fraction	Milled	Not milled	Milled	Not milled
Soluble	35½	52	16	19½
Chloroplast	3½	46	2	22
Residue on filter paper pulp	61	1	82	59

Average results are shown in Table 13. Milling chloroplasts with cellulose has caused more than half of the nitrogen present to adhere to the fibres, whereas simple mixing has resulted in a loss on the fibres of 1 % only. The nitrogen content of the milled residue is 2·5 % compared with 0·1 % in the unmilled residue. The chloroplast fraction is reduced by the milling to a very small proportion and the amount of soluble material resulting from the action of the dilute alkali on the chloroplasts much decreased. Thus the material attached to the fibres is less susceptible to the action of dilute alkali. The loss of

material on the fibre was so large that considerable increases of extraction were to be expected by not remixing chloroplasts for further treatment.

Table 14 sets out the results of two series of experiments to show the effect with leaves. In those marked (*a*) the chloroplast fraction has been mixed with the residue at each step and the whole subjected soaking at pH 8·0. Together with the fact that yields of soluble fraction after milling (columns 6 and 13) seem to be little affected by the presence or absence of chloroplasts, this means that the over-all yield of soluble material is a little reduced by not including the chloroplasts from mincing in the preliminary soak at pH 8·0.

Table 14. *Effect of re-extracting chloroplasts with the fibre and of keeping them separate*

	% extraction of component in leaf													
	Nitrogen							Dry matter						
	Mince		Neutralize		Milled			Mince		Neutralize		Milled		
Species	Soluble	Clp.	Soluble	Clp.	Soluble	Clp.	Residue	Soluble	Clp.	Soluble	Clp.	Soluble	Clp.	Residue
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
(*a*) Chloroplasts returned to fibre for extraction														
Tobacco	44	—	7	—	29	—	20	39	—	3	—	9½	—	48½
Tobacco	37	—	10	—	31	—	22	45½	—	7	—	11	—	36½
Nicotiana glutinosa	36	—	9½	—	12½	15½	26½	51	—	4½	—	5	9	30½
(*b*) Chloroplasts kept separate														
Tobacco	33	18	5	1½	30	6	6½	37	13	3	1	12	8	26
Tobacco	34	20	4	1½	21½	6	13	40	15	2½	1	9½	4½	27½
Nicotiana glutinosa	36	22	4	3	9	8	18	51	10½	2	2	4	4½	26

Clp. = 'chloroplastic'.

to the next extraction. In those marked (*b*) the chloroplasts were kept separate. As was to be expected from the above results, a considerable increase in total extraction over series (*a*) can be observed in series (*b*) experiments. An identical picture is shown in Table 15 from which it can be

Table 15. *Extraction (soluble + chloroplast) when milling is done after or before neutralizing*

% extraction of component in material milled			
Nitrogen		Dry matter	
Milled after neutralizing	Milled before neutralizing	Milled after neutralizing	Milled before neutralizing
Chloroplasts returned to sequence			
63	49	25½	24½
Chloroplasts kept separate			
78	57	42½	35½

seen that whether milling is done before or after neutralization, separating the chloroplasts at each stage increases the total extraction from the fibre. The residue (columns 8 and 15, Table 14) from series (*b*) experiments contains only about half of the nitrogen and dry matter found in the others. The values in columns 2 and 9 will not, of course, be affected, while values only appear in columns 3 and 10 for those experiments in which chloroplasts were separated. It can be seen that failure to replace chloroplasts reduces the yield of soluble fraction by

That the extra amounts of nitrogen and dry matter in the residue from series (*a*) (columns 8 and 15) is not wholly due to the inclusion of the chloroplasts from the milling stage is shown in the *Nicotiana glutinosa* experiments, where mill chloroplasts and residue were estimated separately. The series (*a*) residue is still higher in both nitrogen and dry matter, showing that the effect observed with cellulose fibres also holds good for leaf fibres. The difference would not be expected to be so great for leaf fibres, for filter paper mills with great difficulty and any deleterious effect on the chloroplastic material would be expected to be greatly intensified.

Neutralization after milling. By far the most time-consuming step in the sequence so far described is the adjustment of the fibre to pH 8·0 (6–7 hr.). If the fibre is first milled, pH equilibrium is reached very much more rapidly and the greater part of the soda can be added within 2 hr. (cf. Holden, 1945). The effect on the extraction of this reversal of the order of neutralization is shown in Table 15 where the figures are for total extraction at each step (soluble + chloroplast). From this it can be seen that the order of neutralization has a considerable effect, the extractions being appreciably higher when the fibre is neutralized before milling. This is true whether the chloroplasts are kept separate or returned to the extraction sequence. More serious, the reduction of nitrogen extraction is a good deal more than of dry matter when the fibre is milled before neutralization. It seems unlikely that the

alkaline wash would promote extraction by removing salts not already extracted by the water washing to which the fibre has already been subjected. It is possible that some other inhibitory substance has been removed. However, the most plausible explanation is that increase of pH has lead to extra imbibition of water by the polysaccharide components of the leaf with consequent swelling and softening. Considerable swelling occurs as is shown by settling experiments with washed fibre suspended in a large volume of water. At pH 8·0 the volume occupied by the settled fibre is three or four times that occupied at pH 6·5. Neale (1929, 1930) has shown under more extreme conditions that cellulose in the form of cotton, cellophan and rayon swells considerably in 0·1 M- and perceptibly in 0·01 M-NaOH. Further, Cross & Bevan (1885) observed that cellulose in the green leaf was much less resistant to reagents before it had been dried, which would indicate that swelling of undried leaf fibre would be considerable even when the NaOH concentration was below 0·01 M. The behaviour of the leaf fibre is thus consistent with what might have been expected in mildly alkaline fluids although leaf fibre is a good deal more sensitive than prepared cellulose. The resulting softening of the fibre might well lead to more efficient milling and hence to a higher extraction.

Serial milling. The average extractions of nitrogen and dry matter after more than one milling are shown in Table 16. After each milling, the fibre was adjusted to pH 8·0 and washed twice in the usual way. Series in which neutralization was done before milling and in others in which it was done afterwards are included.

Table 16. *Serial milling and its effect on extraction*

	% extraction of component in fibre			
	Nitrogen		Dry matter	
Extraction process	Milled after neutralizing	Milled before neutralizing	Milled after neutralizing	Milled before neutralizing
Neutralization	15½	—	11½	—
1st milling	63	61	34½	36
2nd milling	8½	19	5½	10½
3rd milling	—	6	—	1½

It can be seen that a second milling is as effective as a preliminary adjustment of pH. Similarly, the over-all extraction for neutralization plus two millings is approximately the same as for three millings. However, the extraction of nitrogen becomes progressively more difficult. Thus the second milling removes 40–50 % of the nitrogen remaining after the first milling, but the third removes only 30 % of that left after the second milling. At this stage, the residue contains only 0·5 % nitrogen on a dry-weight basis and usually represents only 5 % of the nitrogen of the intact leaf.

It would appear from the fact that the ratio % nitrogen extracted/% dry matter extracted is higher in the later millings, that the nitrogen/dry matter ratio in the later millings might be higher than in the earlier ones. That the opposite is true indicates that only a proportion of the residue is affected by the grinding process.

Three millings or two millings subsequent to neutralization are all that are worth while in most cases. The extracts have by this stage become very dilute—only 0·25 g./l., and progressively larger amounts of the structural material of the leaf are broken down with each milling.

Shaking with sand. To investigate the possibility of removing some of the nitrogen remaining in the residues after milling, they were shaken with sand according to the technique of Curran & Evans (1942). Suspensions of fibre residue, 10 mg. dry matter/ml., were shaken for 5 hr. with an equal weight of graded sand on the high-speed shaking machine (225 strokes/min.). The results were disappointing. Approximately 10 % of the nitrogen in the fibre residue was brought off a fibre containing 0·6–0·7 % N. With fibres of higher N content, more success was attained, but these are more easily dealt with by the triple-roller mill. The two techniques, therefore, appear to have approximately the same limits of extraction.

DISCUSSION

The final sequence of extraction processes elaborated during this investigation is: mincing and washing, milling twice or thrice in the triple-roller mill and extraction of this material with dilute NaOH at pH 8·0. By this means it is possible consistently to separate 90–95 % of the nitrogen of tobacco leaves from the insoluble residue. On occasions the extraction rises to 99 %. At the same time, approximately 80 % of the total dry matter is also dispersed.

So far as can be judged, the most important factors giving maximum extraction are: the introduction of as much shearing stress into the grinding as possible, the use of extractions at a pH as high as possible without damaging the proteins, keeping the salt concentration low and separating the chloroplast fraction from the residue at each stage. The material must not be frozen at any stage. Any departure from these conditions lowers the yield from tobacco leaves. Thus, freezing leads to considerable denaturation when losses of 30 % may occur (Table 5).

Increasing the pH of extracting fluids from 6 to 8 has been shown to increase the extraction of nitrogen

and dry matter by a factor of three (Table 6). The work of Lugg (1939) indicated that even better results could be obtained by increasing the pH still further and this has been confirmed here (Table 11). The problem of setting an upper limit to the pH of extracting fluids is a difficult one. If the properties of a particular protein are known the fixing is more or less automatic, but when, as here, an attempt is made to extract all proteins with a minimum of damage some more or less arbitrary general level must be fixed. This will be determined by the more sensitive components of the leaf proteins and a consideration of the properties of certain viruses and enzymes already mentioned has led to the fixing of the pH at 8·0.

Many extracting solutions such as buffers and calcium reagents, which would appear to offer definite advantages, all suffer from the defect that they increase the salt concentration. The degree of dispersion and the ease of extraction of the leaf proteins are extraordinarily sensitive to small concentrations of salts (Table 10). Most satisfactory results have therefore been obtained by avoiding salts, even in the form of buffers, and using only dilute solutions of sodium hydroxide to adjust the pH of extraction fluids. This experience is in direct contradiction to the suggestion of Vickery (1945). He suggests, presumably from analogy with the behaviour of seed proteins, that the difficulty experienced by Chibnall in dispersing the 'cytoplasmic' protein after ether cytolysis was due to removal of the proteins from a medium of high to one of low ionic strength when the cell was broken up. He suggests, too, that the extra efficiency of 'used' ether-water was due to small amounts of salts introduced during its previous history. In the present experiments it has been shown that small quantities of salts, either at the natural pH of the leaves or at pH 8·0, decrease the amount of nitrogen and dry matter extracted. The properties of the green leaf proteins of tobacco are therefore considerably different from those of many seed proteins whose solubility is greatly increased by small concentrations of salts.

The most important factor in the grinding process is the amount of shearing stress to which it subjects the leaves. Thus, in the comparison of the mincer and the food cutter, it was found that the size of the leaf fragments was very similar, yet the extraction from minced leaves was higher (Table 1). The higher efficiency of the mincer appears to be due to the crushing and bruising to which it subjects the leaves. Among the machines used for the reduction of minced fibre, the order of increasing amounts of shearing during grinding is approximately: Latapie, end-runner, edge-runner and triple-roller mills, and this again is the order of increasing extraction from the ground products. In the Latapie, the emphasis is upon a cutting action with incidental shearing occurring at the places where the cut occurs. Microscopic examination shows fragments having many cells with apparently undisturbed contents. At the other end of the scale, shearing and pressure in the roller mill tears apart the tough cell walls, flattens out the fragments and scours off the cell contents. This picture is supported by the results of several millings, when it is still possible to separate 30 % of the nitrogen from fibre containing only 0·7 % N on a dry-weight basis, together with very small amounts (approx. 4 %) of the dry matter. The shearing action of the mill must be 'peeling off' from each particle, a layer comparatively rich in nitrogen rather than breaking up the particles and dispersing the fragments. Leaves, in general, appear to be very susceptible to shearing stresses. Thus, Phillis & Mason (1937) found that such a small amount of shearing as that induced by 'gently rubbing between finger and thumb' the leaves of the cotton plant, was sufficient to increase the amount of sap, which could be pressed out in a hydraulic press, by a factor of four and to reduce the pressure necessary to express it from 4000 lb./sq.in. to something too small to show on the gauge.

Increasing the alkalinity of extracting fluids, besides promoting solution of the proteins, increases the efficacy of the shearing action. This probably occurs through increased imbibition of water by cell-wall materials which leads to swelling and softening. Another possible promoting action of a high pH is its liberation of pectase (Holden, 1945) which attacks the leaf pectin and would be expected to weaken the middle lamella and make grinding easier. The opposite effect is seen when the moisture content of fibre is reduced preparatory to milling. Then, the extraction of dry matter tends to decrease more rapidly than the extraction of nitrogen (Table 3); the proteinaceous parts of the leaf have a greater water-holding power than the 'structural' parts.

As has been noted by many workers, leaf proteins are difficult to disperse and bring into solution. Thus from Table 14 it can be seen that approximately 30 % of the total nitrogen separated from the tobacco leaf remains in the chloroplast fraction. By regrinding this with fibre, nearly one-third of it can be dispersed so that it does not sediment in a field of 1500 × gravity applied for 15 min. The shearing action of the mill is thus having an effect other than that of scouring cell contents off the wall. It is also breaking up and dispersing those contents after the manner of a colloid mill.

However, the chloroplasts are the most resistant cell constituents, probably because of their high lipid content, and show up most clearly the disadvantages of the triple-roller mill. Once separated from the bulk of the leaf residue, they must be kept separate for maximum extraction. Any small gain

in soluble material obtained by regrinding them with the remaining fibre is more than offset by the loss in extraction caused by the fixation of chloroplasts to the residue (Table 14). This fixation is sufficiently firm to resist elution by NaOH at pH 8·0 and may represent considerable amounts of nitrogen, as in the filter-paper experiments (Table 13). A similar result has been obtained by Bawden & Pirie (1944) with tomato bushy stunt virus. As the chloroplasts are the most difficult to disperse it seems likely that a considerable proportion of the nitrogen remaining on the fibre will be of this origin. However, that virus also may be caused to adhere indicates that other cell constituents are represented among this firmly adhering material. Thus it would appear that some degree of equilibrium between material on the fibre and that dispersed is reached at each grinding.

The major constituent of the residue is carbohydrate. Approximately 75 % of the dry matter of the residue estimates as carbohydrate by the orcin method of Pirie (1936). The nitrogen content is generally of the order of 1 %, so that approximately 6 % of the residue could be accounted for by protein. Approximately 4 % is lipid material (soluble in acid-alcohol-ether) and 15 % is ash. According to Kissling (1926), the cellulose content of the tobacco leaf is 10–15 % of its dry matter, but it is unlikely that all of this is concentrated in the final residue. Appreciable quantities of fine cell-wall debris, often with protein attached, find their way through the cloth during squeezing and contaminate the chloroplast fractions. As well as cell-wall material, the chloroplast fractions contain starch, particularly in summer. There is some evidence that other formed elements of the cell, such as mitochondria are also present.

It has been emphasized that the definition of 'soluble' adopted in this work has been material which does not sediment in 15 min. in a centrifugal field of approximately 1500 × gravity. Most of the solutions thus obtained are deep green and contain particles visible under the microscope. These are largely plastid fragments varying in size from 5μ diameter down to the limits of resolution, but starch fragments and minute cell-wall debris are also present. Some of this material can be removed by prolonged spinning at 1500 × gravity, and the bulk of it sediments at 8000 × gravity. There is little evidence whether or not the protein fraction of the sedimentable material consists wholly of plastid fragments (chromoprotein) and other similar formed elements. The observation that tomato bushy stunt virus is attached to chromoprotein by milling and only liberated therefrom by the action of trypsin (Bawden & Pirie, 1944), suggests that other soluble cell constituents are attached in a similar manner. This attachment of soluble material to chromoprotein is a phenomenon similar to the attachment of chromoprotein to cell debris. It indicates a certain amount of 'scrambling' of the cell contents and is the greatest disadvantage of the milling process. Bawden & Pirie suggested that it was due to momentary heating as the leaf material passed between the rollers.

However, in spite of these defects, the high extractions here reported have been obtained under conditions less likely to cause grave alteration to the proteins than any so far mentioned in the literature. Much valuable data should result from further study of the products obtained.

It would seem impossible to find any means of grinding which is as efficient in dispersing the leaf proteins and yet at the same time does not cause this local heating with consequent alteration of proteins and their attachment to one another and the cell debris. Further, with 95–98 % extraction, the limit of removal in the form of solutions sufficiently concentrated for easy manipulation is almost reached. The problem then is one of finding a means of reduction of the leaf more mild than grinding and one which will not leave so much nitrogen attached to a residue. The most hopeful solution to the first would appear to be the use of enzymes. Using purified pectinases and cellulases, the cell contents should be altered only to the extent that extra- and intracellular environments are different. The contents would not be dispersed to the same degree as would be obtained from the shearing action of the mill. On the other hand, most of the formed elements of the cell would be intact and an opportunity presented of studying not only the types of protein present, but their spatial distribution in the cell.

SUMMARY

1. A process for extracting 90–95 % of the nitrogenous material from tobacco leaves is described.

2. The steps in this process are: preliminary mincing and washing, grinding in a triple-roller ointment mill, extraction with dilute sodium hydroxide at pH 8·0, repetition of grinding and extraction.

3. The triple-roller mill is effective because of the rubbing to which it subjects the leaves.

4. The salt concentration must be minimal for best extraction; buffers and compounds preventing the precipitation of Ca-complexes reduce extraction.

5. Regrinding of separated chloroplastic material with partially extracted leaf fibre decreases rather than increases the extraction of protein.

6. Grinding under Lugg's (1939) conditions was found to be less effective with tobacco leaves than the process described here.

7. All grinding methods have the disadvantage that they cause some degree of alteration of the

proteins. Nevertheless, the high extractions here reported have been obtained under conditions less likely to cause grave alteration to the proteins than any so far reported in the literature.

8. Approximate calculations based on the extraction give the average protein content of tobacco leaves to be 15 % of the dry matter, of which 95 % is extractable.

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Oxidations in Acetobacter

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The question of the intermediary stages of the bacterial oxidation of acetic acid is one of the major unsolved problems in the intermediary metabolism of bacteria. Quastel & Webley (1941) showed that aneurin and co-carboxylase can increase the rate of oxidation of acetic acid in strain number 4759* of the National Collection of Type Cultures, but other evidence indicated that pyruvate is not an intermediate in acetate oxidation.

In *Acetobacter*, acetic acid is an end-product under some conditions, especially when ethanol is the substrate (Brown, 1886), but it is readily oxidized under other conditions (Tosic, 1942). This observation was studied in detail, as it was thought that an organism in which incomplete oxidations occur might be specially suited for work on intermediary metabolism.

* This organism is listed as a '*Propionibacterium*', but it was shown by Krebs & Eggleston (1941) that the organism does not possess the generic characteristics of *Propionibacterium*.

EXPERIMENTAL

Material and methods

The organism. The original strain of *Acetobacter turbidans* (Cosbie, Tosic & Walker, 1942) was used in most experiments.

The cells were grown on a basal medium to which suitable organic substrates, such as glucose, glycerol, ethanol, and sodium lactate, were added. The basal medium contained: 5·0 g. 'Difco' yeast extract, 2·0 g. $(NH_4)_2SO_4$, 2·0 g. KH_2PO_4, 0·2 g. $Na_2HPO_4.2H_2O$, 2·0 g. $MgCl_2.6H_2O$ in 1000 ml. tap water. Larger quantities of the organism were grown in Roux bottles containing 100 ml. basal medium, substrates and 2% agar, at the optimum temperature for growth (25°). The inoculum per 100 ml. medium was a 2 ml. portion of a 24 hr. old test-tube culture grown in the basal medium. After 40 hr. incubation (unless otherwise stated) cells were washed off the agar with distilled water, centrifuged, resuspended in water, centrifuged again and finally suspended in a small volume of water. The concentration of the 'stock suspension' was determined by drying 1 ml.

at 100° and weighing. Stock suspensions usually contained about 20 mg. cells (dry matter)/1 ml., and were stored at about 2° (see below).

Yields of cells. The effects of substrates and of the duration of the incubation on yields are summarized in Table 1.

Table 1. *Effect of the addition of substrates to the basal medium and of the period of incubation on the yield of bacteria*

(The data refer to one Roux bottle containing 100 ml. medium.)

Substrate added to basal medium	Yield of cells (mg. dry matter) after incubation at 25° for		
	24 hr.	40 hr.	48 hr.
None	5	7	8
1% glucose	27	40	46
1% glycerol	22	48	67
1% ethanol	40	76	80
1% Na lactate	75	110	130
0·5% Na lactate + 0·5% ethanol	130	150	160

Addition of ethanol, glucose, glycerol or Na-lactate greatly increased the yield of cells; the highest yield was obtained when the medium contained ethanol and lactate together. During growth, the former substrate yields acetic acid and the latter $NaHCO_3$, and as a result the medium retains a hydrogen ion concentration compatible with rapid growth longer than other substrates. An analogous 'buffer' effect has been recorded by Grey (1924) for *Esch. coli*.

Manometric procedure. The oxidative activity of cell suspensions was measured in the usual way in Warburg manometers at 25° in the presence of 0·1 M-phosphate buffer at pH 6·8. Conical cups (about 20 ml. capacity) with a centre well were used; substrates were added from the side arm.

Oxidative activity of cell-suspensions. The rate of oxidative metabolism of freshly harvested cells varied according to the age of the culture, the chemical nature of the medium on which the cells were grown and on the substrate of oxidation. The data in Table 2 indicate that the older

Table 2. *The influence of age of culture of* A. turbidans *on* Q_{O_2}

(Q_{O_2} in 0·1 M-phosphate buffer at pH 6·8 in the presence of ethanol; 25°.)

Additions to basal medium	Q_{O_2} at different ages of culture			
	24 hr.	40 hr.	48 hr.	72 hr.
1% glucose	84	*100*	54	5
1% glycerol	99	*154*	146	93
1% ethanol	314	*332*	196	141
1% Na lactate	87	*91*	72	5
0·5% ethanol + 0·5% Na lactate	*210*	201	180	120

cultures contained less active cells than the younger ones. They also show that some enzymes (e.g. those concerned with the oxidation of ethanol) were partially adaptive. Similar results were obtained when glucose, glycerol or lactate was the substrate of oxidation. As the cells grown on the medium containing lactate and ethanol together gave a high Q_{O_2} (as well as a high yield of cells) this medium was used throughout for further experiments, with a period of incubation of 40 hr.

The oxidative activity of cell-suspensions showed little loss on storage up to 12 days at about 2°. After 8 days' storage the fall of Q_{O_2} amounted to 10, 14 and 15% when tested on glucose, ethanol and lactate as substrates respectively.

Cells suspended in phosphate buffer alone, serving as controls, gave negligible if any oxygen uptake.

RESULTS

Oxidation of various substances. It will be seen from Table 3 that the number of substrates oxidized in *A. turbidans* is limited. The organism oxidized *n*-propanol but not *iso*-propanol; of all the sugars tested only glucose was attacked. Neither glycollic nor oxalic acid, which might be expected to be intermediates in the oxidation of acetic acid, was oxidized. The oxidation of ethanol, *n*-propanol, formate, lactate and pyruvate was very rapid, whilst that of the other oxidizable substances (Table 3) was relatively slow. All substrates, except ethanol, propanol and formate, showed a lag period during which the rate of oxidation gradually increased (Fig. 1). The high rate of formate oxidation suggests the possibility that this substance might be an intermediate in acetate oxidation.

Further experiments in which the oxygen uptake was measured until it ceased showed that the total oxygen consumption was 90–95 % of that required for complete oxidation in the case of formate, acetate, pyruvate and lactate. When ethanol was the substrate one molecule of oxygen was rapidly absorbed. This was followed by the slower uptake of two further molecules of oxygen as required for complete oxidation. Propanol absorbed one molecule of oxygen. In the case of glucose half a molecule of oxygen was rapidly absorbed. This was followed by the very slow uptake of about 80 % of that necessary for complete oxidation.

The lag period in the oxidation of acetate. The factors influencing the oxidation of acetic acid have been studied and attempts have been made to elucidate the cause of the lag period. At pH 5·9, 6·8 and 7·8 there was no difference in the length of the lag period or in the rate of oxidation of acetate (Table 4). The lag period was little affected by the concentration of acetate. Table 5 shows that the lag period was shortened and the initial Q_{O_2} raised by an increase of the density of cell suspensions. Storage of cell suspensions at about 2° prolonged the lag period as shown in Table 6.

Effect of various substances on the lag period in acetate oxidation. A relatively small quantity (1 μmol.) when added to a larger amount of acetate (5 μmol.) had a pronounced effect on both the reduction of the lag period and the rate of acetate

Table 3. *Oxidation of various substrates in* A. turbidans

(Main compartment: 1 ml. cell suspension; 1 ml. 0·1M-phosphate buffer, pH 6·8; 0·9 ml. H_2O. Side arm: 0·1 ml. substrate (the acids added in form of neutral Na-salts). Centre cup: 0·2 ml. 2N-NaOH; air; 25°.)

Substance tested	Added amount of: Substrate (μmol.)	Cell (mg.)	μl. O_2 absorbed after (min.): 10	20	40	60
Formate	20	4·0	90	142	170	178
Acetate	5	5·0	0	4	16	27
Glycollate	5	4·0	0	0	0	0*
Propionate	5	5·0	0	0	0	0*
dl-Lactate	5	3·7	48	132	237	254
Pyruvate	5	3·7	15	43	153	223
n-Butyrate	5	5·0	0	0	0	0*
Oxalate	5	4·0	0	0	0	0*
Malonate	5	4·0	0	0	0	0
Succinate	5	5·0	7	26	76	122
dl-Malate	5	3·7	0	0	0	5
l-Malate	1	5·0	0	0	0	6
Fumarate	1	5·0	5	14	48	67
α-Ketoglutarate	5	3·7	0	0	28	41
Citrate	5	4·0	0	0	0	0*
*l-iso*Citrate	5	4·0	0	0	0	0*
Methanol	5	5·0	0	0	0	0*
Ethanol	5	1·3	40	61	87	114†
n-Propanol	5	1·3	59	91	99	100†
*iso*Propanol	5	5·0	0	0	0	0
Glycerol	5	5·0	7	34	112	197
α-Glycerophosphate	5	5·0	0	0	0	0
β-Glycerophosphate	5	5·0	0	0	0	0
d-Sorbitol	5	5·0	0	0	0	0*
d-Mannitol	5	4·0	0	0	0	0*
Dulcitol	5	4·0	0	0	0	0*
i-Inositol	5	4·0	0	0	0	0*
Formaldehyde	1	5·0	2	7	10	17
d-Glucose	5	4·0	31	65	118	155
Galactose	5	4·0	0	0	0	0*
Mannose	5	4·0	0	0	0	0
Fructose	5	4·0	0	0	0	0
Maltose	5	4·0	0	0	0	0*
Lactose	5	4·0	0	0	0	0*
Sucrose	5	4·0	0	0	0	0*

* No O_2 uptake after 3 hr.

† O_2 uptake values lower than theoretically expected, owing to evaporation of the substrates from the side arm during equilibration before mixing.

Table 4. *Effect of* pH *on acetate oxidation in* A. turbidans

(Main compartment: 1 ml. cell suspension (5 mg. dry-cell matter). 1 ml. 0·1M-phosphate buffer; H_2O to 3 ml. vol. Side arm: 0·1 ml. 0·05M-acetate; centre cup: 0·2 ml. 2N-NaOH; air; 25°.)

Time (min.)	μl. oxygen uptake: pH 5·9	pH 6·8	pH 7·8
10	0	1	1
20	2	5	5
30	6	9	9
60	20	21	22

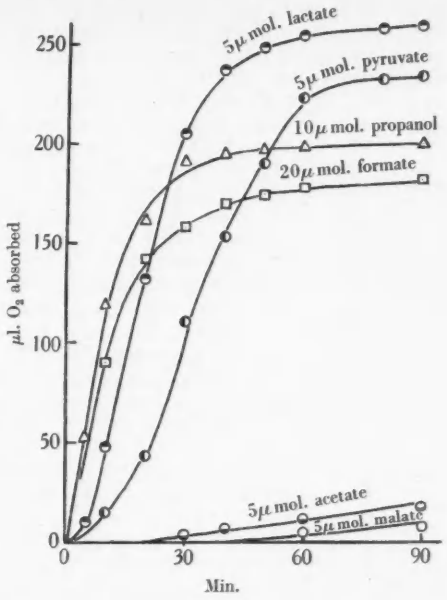

Fig. 1. Lag period in oxidation of some substrates in *A. turbidans*. Contents of cups: 1 ml. 0·1M-phosphate buffer, pH 6·8; 1 ml. fresh cell suspension (=4 mg. dry weight in case of acetate, formate and propanol; in other cases = 3·7 mg.); 0·2 ml. substrate solution; 0·8 ml. water; air; temp. 25°.

Table 5. *Effect of the amount of cell matter on acetate oxidation in* A. turbidans

(Condition as in Table 4, but pH 6·8. The quantity of cells stated was suspended in 3 ml.)

Time (min.)	μl. O_2 absorbed by: 1 mg.	5 mg.	10 mg.
10	0	1	2
20	1	5	12
30	1	9	20
60	3	21	53

Table 6. *Effect of storage of cell suspensions on the lag period in acetate oxidation in* A. turbidans

(Conditions as in Table 4, but pH 6·8.)

Storage at 2° (days)	μl. O_2 absorbed after: 10 min.	20 min.	30 min.	60 min.
2	0	7	16	52
5	0	6	12	37
12	0	0	0	6

oxidation. It can be seen from curves in Fig. 2 that the O_2 uptake due to acetate and lactate, when added together, is very much more rapid than the O_2 uptake of the suspensions to which the substrates are added separately. After 30 min., for example, the cell suspension with acetate alone absorbed no measurable amount of oxygen, but when 1 μmol. of lactate was present 216 μl. O_2 were taken up. The complete oxidation of the added lactate required 67·2 μl. O_2. The excess—149 μl.—accounts for the complete oxidation of 67 % of the added acetate. A similar accelerating effect was obtained when 0·1 μmol. of lactate was added together with 5 μmol. of acetate.

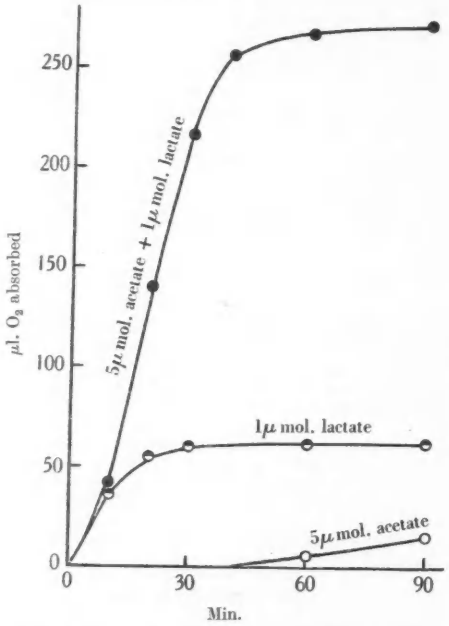

Fig. 2. Effect of lactate on acetate oxidation in *A. turbidans*. Contents of cups: 1 ml. cell suspension (5 mg. dry weight) stored for 11 days at about 2°; 1 ml. 0·1 M-phosphate buffer, pH 6·8; 0·2 ml. substrate solution; made up to 3 ml. with water; air; temp. 25°.

Similar effects on acetate oxidation were observed when lactate was replaced by pyruvate, succinate, *l*- or *dl*-malate, fumarate, α-ketoglutarate, glycerol, glucose, formate or formaldehyde. Representative curves showing the effect of α-ketoglutarate and formate on acetate oxidation are given in Figs. 3 and 4 respectively. Experiments with washed suspensions of another species, *Acetobacter mobile*, confirm the enhancing effects of lactate and other substrates on acetate oxidation.

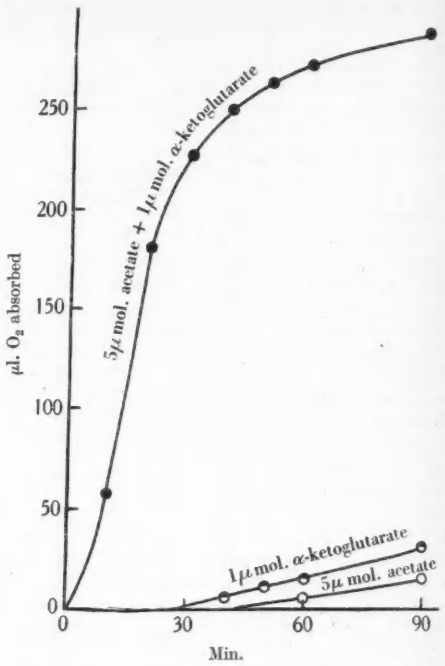

Fig. 3. Effect of α-ketoglutarate on acetate oxidation in *A. turbidans*. Conditions as given in Fig. 2.

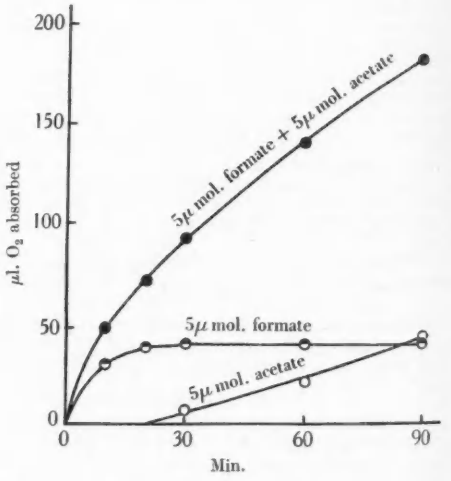

Fig. 4. Effect of formate on acetate oxidation in *A. turbidans*. Conditions as given in Fig. 2.

Quastel & Webley (1942) recorded somewhat similar observations; they showed that strain no. 4759 of National Collection of Type Cultures, after incubation with hexosediphosphate, pyruvate, fumarate, glycerol or fructose, oxidized acetate at an increased rate.

The oxidation of substances, other than acetate, which showed a pronounced lag period (e.g. succinate, fumarate, α-ketoglutarate, glucose) was not increased by the addition of lactate. The substances in Table 3, which were not oxidized when added alone, remained unaffected in the presence of lactate. Neither ethanol nor propanol, nor any of the non-oxidizable substances (Table 3), when added together with acetate accelerated its oxidation.

The fact that so many different substances, including such a simple compound as formate, have a similar accelerating effect, makes it unlikely that the carbon skeleton of these substrates provides directly an intermediate which is essential for the oxidation of acetate. It is more probable that the process of oxidation of the second substrate leads to the formation of a substance essential for acetate oxidation.

Effect of H_2O_2 *on acetate oxidation.* It seemed possible that H_2O_2 might be formed in small quantities in the course of oxidation of the second substrate, and induce acetate oxidation through a peroxidase. Different concentrations of H_2O_2 (equivalent to 5–100 μl. O_2) were added together with acetate in order to test this hypothesis, but it was found that the addition did not accelerate the oxidation of acetate. The fact that some substances reduce the lag period of acetate oxidation without undergoing, in the early stages, a measurable degree of oxidation (e.g. α-ketoglutarate, Fig. 3) is also contrary to the peroxidase hypothesis.

Effect of vitamin B complex components on acetate oxidations. An alternative explanation of the lag period would be the hypothesis that for the oxidation of acetate a specific catalyst is required, which may be formed under the influence of one of the substances reducing the lag period. To replace the effect of these substrates, components of the vitamin B complex, which are known to be catalysts in bacterial oxidation of acetate (see especially Quastel & Webley, 1942) were added to acetate. The substances and the quantities added to 4 ml. suspensions were: aneurin (10 μg.), co-carboxylase (10 μg.), riboflavin (10 μg.), pyridoxin (10 μg.), nicotinic acid and its amide (20 μg.), biotin (10 μg.), pantothenate (10 μg.), inositol (20 μg.) and *p*-aminobenzoic acid (20 μg.). In view of the finding of Quastel & Webley (1942) that K^+ and Mg^{++} increase oxidation of acetate in the presence of vitamin B_1 in strain no. 4759, the tests were conducted in the presence and absence of these ions. None of the components of the vitamin B complex accelerated the oxidation of acetate, either singly or in various combinations, one of which included all the substances. The addition of K^+ or Mg^{++} did not accelerate oxidation of acetate in these experiments to any appreciable extent.

Effect of adenosinetriphosphate (ATP) on acetate oxidation. It was found (Table 7) that addition of ATP increased the rate of acetate oxidation in *A. turbidans*, but the accelerating effect was not as great as that of simpler organic substances, e.g. lactate. The preliminary incubation of vitamin B-deficient cells of strain no. 4759 was shown by Quastel & Webley (1942), to cause a small but

Table 7. *Effect of adenosinetriphosphate (ATP) on acetate oxidation in* A. turbidans

(Conditions as in Table 4, but pH 6·8. ATP added as Na salt.)

	μl. O_2 absorbed with substrate			
Time (min.)	ATP (1 mg.)	Acetate 5 μmol.	Acetate 5 μmol. + 1 mg. ATP	Acetate 5 μmol. + 0·1 mg. ATP
10	0	1	2	1
20	0	3	10	7
30	0	7	18	12
60	0	20	40	29
120	0	55	95	73

Table 8. *Effect of ATP on acetate oxidation in* A. turbidans *in presence and absence of pyruvate*

(Conditions as in Table 4, but pH 6·8, and the cell suspension was stored at about 2° for 10 days before use.)

	O_2 uptake (μl.)						
Time (min.)	5 μmol. acetate + 1 μmol. pyruvate + 1 mg. ATP	5 μmol. acetate + 1 mg. ATP	5 μmol. acetate + 1 μmol. pyruvate	1 μmol. pyruvate + 1 mg. ATP	5 μmol. acetate	1 μmol. pyruvate	1 mg. ATP
10	22	0	22	12	0	6	0
20	106	6	97	33	0	26	0
30	156	13	145	35	1	30	0
60	242	32	224	38	10	35	0
120	266*	65	262*	38*	29	36*	0

* No further O_2 uptake occurred.

definite acceleration of acetate oxidation. The effect of ATP on acetate oxidation in our organism was also tested in the presence of vitamin B_1 and other members of the vitamin B complex, but it was not greater than that obtained in the presence of ATP alone. The oxygen uptake on the addition of acetate together with a little pyruvate was slightly increased by the further addition of ATP (Table 8); thus the effect of ATP appears to be additive in *A. turbidans*.

DISCUSSION

Whilst it has not been possible to elucidate the intermediary stages of acetate oxidation in *Acetobacter*, some factors influencing the oxidation of acetate have come to light:

(1) The presence of small quantities of certain simple organic substances (lactate, pyruvate, glycerol, fumarate, succinate, malate, α-ketoglutarate, glucose, formate, formaldehyde) increases the rate of acetate oxidation and diminishes, or almost abolishes, the lag period in *Acetobacter*. The effective substances have the common property of undergoing oxidation, though at very different rates, in the organism, but not every substance which is oxidizable has an accelerating effect; ethanol and propanol are rapidly oxidized yet they do not affect acetate oxidation.

(2) ATP (but not any of the members of the vitamin B complex tested either alone or in various combinations) also increases the rate of acetate oxidation and reduces the lag period, but the effect is smaller than that of any of the above-named substances. ATP caused a slight additional increase in the rate of oxidation of the pyruvate-acetate mixture.

Effects resembling those described in this paper were found by Quastel & Webley (1942) in strain no. 4759, as already quoted.

To explain the enhancing effects of relatively simple substances we may assume that: (*a*) the carbon skeleton of the added substrates provides directly an intermediate which is essential for the oxidation of acetate, or (*b*) that the added compounds (or their breakdown product) give rise in the presence of acetate to the formation of an intermediate necessary for the oxidation, or (*c*) that the simple substances cause the activation of one of the enzymes essential for acetate oxidation. The fact that so many different substances, of which some are oxidized at a very low rate, have a similar enhancing effect makes it unlikely that explanation (*a*) is the correct one. Assumption (*b*) is more probable, although the fact that some substances reduce the lag period of acetate oxidation without, in the early stages, being themselves measurably oxidized (e.g. α-ketoglutarate) is not in agreement with this hypothesis. Furthermore, the increased oxidation is produced even by very small quantities of the second substrate (e.g. 0·1 μmol. lactate). It is more likely, therefore, that the effect is of the type suggested under (*c*), so that the last assumption may prove more useful as a working hypothesis.

SUMMARY

1. Conditions have been described for the preparation of washed cell suspensions of *A. turbidans* suitable for metabolic experiments. The best yield of highly active cells was obtained when the medium contained 0·5 % ethanol and 0·5 % lactate as substrates. The acid produced from ethanol is neutralized by bicarbonate formed from lactate and thus a pH compatible with rapid growth is maintained for a relatively long period.

2. The oxidation of various substances by a washed cell suspension was examined. Ethanol, propanol, lactate, pyruvate, glycerol, glucose, formate and a number of other substances (Table 3) were found to be oxidized. Methanol, *iso*propanol, glycollate, propionate and butyrate are among the substances which were not oxidized. In the case of formate, formaldehyde, lactate and pyruvate the oxidation was almost complete. Ethanol was rapidly oxidized to acetic acid; acetate was slowly oxidized almost to completion. Propanol was oxidized to propionic acid. The oxidation of glucose was also incomplete.

3. Formate, ethanol and propanol were oxidized without a lag period. Other substances show lag periods of various lengths, e.g. with acetate as substrate, 10–60 min. The duration of the lag period increased with the time of storage of cells and dilution of the suspension.

4. The lag period of acetate oxidation was greatly reduced by the addition of small quantities of lactate, pyruvate, glycerol, succinate, malate, fumarate, α-ketoglutarate, glucose, formate and formaldehyde. The same substances increased the rate of acetate oxidation. The lag period was also somewhat reduced by adenosinetriphosphate.

5. The mechanism of the effects of various substances on the lag period and on the rate of oxidation of acetate is discussed.

The author wishes to express his thanks to Prof. H. A. Krebs for his constant help and advice.

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The Metabolism of Sulphonamides

1. THE METABOLISM OF METANILAMIDE (*m*-AMINOBENZENESULPHONAMIDE) IN THE RABBIT

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The present series of investigations was undertaken with a view to ascertaining whether the metabolic fate of the sulphonamide drugs, particularly in regard to their oxidation, is in any way related to their antibacterial activity or to their toxic effects.

The first compound selected for study was metanilamide, for preliminary experiments had shown that, in the rabbit, it underwent appreciable oxidation and therefore there was a reasonable chance of isolating its oxidation product in a state of purity. Furthermore, the procedure for the isolation of oxidized metanilamide could serve as a model for the more difficult task of isolating the oxidation product of sulphanilamide. Metanilamide is also a sulphonamide which is easily synthesized.

It may be pertinent at this point to refer briefly to some of the biological properties of metanilamide. To mice it is the least toxic of the three isomeric aminobenzenesulphonamides (Laug & Morris, 1939; Dyke, 1937). Tréfouel, Tréfouel, Nitti & Bovet (1937) found it to be therapeutically inactive against streptococcal infections in mice, but in synthetic media devoid of sulphonamide antagonists, Wyss, Strandskov & Schmelkes (1942) found it capable of inhibiting the respiration of several bacteria as effectively as did sulphanilamide. Sevag, Henry & Richardson (1943) have shown that, like orthanilamide and sulphanilamide, it inhibits the carboxylase activity of *Escherichia coli*. Henry (1943) thinks that metanilamide and orthanilamide, despite the fact that they have little or no therapeutic value, may 'have been too hastily regarded as having no fundamental action akin to sulphanilamide'.

No previous study of the fate of metanilamide has been recorded.

METHODS

Animals. Rabbits, maintained on a constant diet of cabbage and Lever's cubes, were used throughout these experiments.

Ethereal sulphate output was determined according to Williams (1938).

In all experiments in this series of papers, where the increase in ethereal sulphate output after feeding sulphonamides has been determined, the rabbits used were maintained on a constant diet for at least a week before the feeding of the sulphonamide. In this way the normal output of ethereal sulphate became steady (cf. Williams, 1938). On a diet of 100 g. cabbage + 100 g. Lever's cubes/day, the normal output of ethereal sulphate in the average rabbit (2–3 kg.) is about 20–40 mg./day. This diet was used in most experiments. Occasionally a diet of 200 g. cabbage/day was used and here the normal ethereal sulphate output was about 10–30 mg./day.

'Free' and 'total' metanilamide were determined in diluted urine (1/50) according to the colorimetric diazo method of Bratton & Marshall (1939), using *N*-(1-naphthyl)-ethylenediamine hydrochloride (British Drug Houses Ltd.) as the coupling agent, colour measurement being made with a 'Spekker' photoelectric absorptiometer.

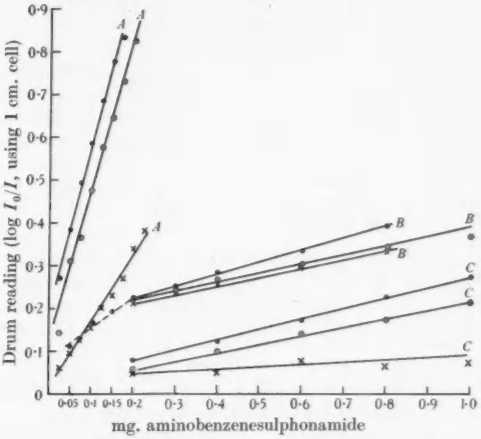


Fig. 1. The relation between drum reading on the 'Spekker' photoelectric absorptiometer and amount of aminobenzenesulphonamide in aqueous solution (using 1 cm. cells and colour filter, Ilford no. 603). *A* are the curves with *N*-(1-naphthyl)-ethylenediamine as coupling agent. *B* are the curves with *N*-β-sulphatoethyl-*m*-toluidine as coupling agent. *C* are the curves with dimethyl-α-naphthylamine as coupling agent. ●—● sulphanilamide. ⊙—⊙ metanilamide. ×—× orthanilamide. •–•–• the common curve for the three sulphonamides in amounts less than 0·2 mg., the coupling agent being sulphatoethyltoluidine.

During this part of the work a comparison was made of the relative usefulness of three coupling agents, namely dimethyl-α-naphthylamine (Marshall, Emerson & Cutting, 1937), *N*-β-sulphatoethyl-*m*-toluidine (Rose & Bevan, 1944) and *N*-(1-naphthyl)-ethylenediamine. Fig. 1 shows that the last is the most sensitive and for our purpose it appeared

to be the most useful for estimating sulphanilamide, metanilamide and orthanilamide in urine. The curves in Fig. 1 were obtained when the total volume of coloured solution was 25 ml. with a 1 cm. cell and Ilford colour filter no. 603 (blue-green). It should be noted that in the case of *N*-*β*-sulphatoethyl-*m*-toluidine, the reagent has an increased sensitivity for amounts of all three aminobenzenesulphonamides less than 0·2 mg. A further increase in the sensitivity of this reagent could, of course, be achieved if a larger cell (3 cm.) had been used.

Preparation of metanilamide. Metanilamide was prepared either by reduction of *m*-nitrobenzenesulphonamide (from *m*-nitrobenzenesulphonic acid, British Drug Houses Ltd.) in ethanolic NH_4OH with H_2S, or from sodium metanilate (British Drug Houses Ltd.) according to Jacobs & Heidelberger (1917).

RESULTS

Quantitative determinations

Ethereal sulphate. The output of ethereal sulphate in rabbits receiving metanilamide is given in Table 1.

Table 1. *The excretion of ethereal sulphate after ingestion of metanilamide*

Rabbit no.	Dose of metanilamide (g.)	Dose of metanilamide (mg./kg.)	Increase in output of ethereal sulphate (mg. SO_3/kg.)	Metanilamide excreted as ethereal sulphate (% of dose)
1	0·769	512	53·7	23·1
2	0·821	498	46·8	20·1
4	1·119	497	45·1	19·4
2	0·650	361	38·0	23·3

In the calculation of the percentage of metanilamide excreted as an ethereal sulphate it is assumed that one molecule of ethereal sulphate is formed from one of metanilamide. The isolation of a monohydroxymetanilamide (see below) showed this assumption to be correct.

'Free' and 'total' metanilamide. The results are given in Table 2. Some explanation, however, is necessary regarding the meaning of these results.

Table 2. *The excretion of 'free' and 'total' metanilamide by the rabbit*

Rabbit no.	Dose of metanilamide (mg./kg.)	% of dose excreted in 2 days 'Free'	'Total'	Apparent acetylation
1	250	29	80·5	51·5
2	250	47	75	28
4	250	50	68	18
8	250	46	81·5	35·5
5	100	35	81	46
45	100	3·5	33	30
46	100	36	68	32
47	100	14·5	90	75·5

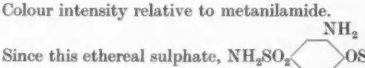
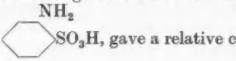
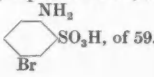
The term 'free' metanilamide includes all compounds excreted which can be diazotized and coupled with naphthylethylenediamine before acid hydrolysis. These include unchanged metanilamide and the ethereal sulphate and glucuronide of 3-amino-4-hydroxybenzenesulphonamide (4-hydroxymetanilamide) (see Table 3). The amount of free metanilamide excreted must be small for the material extractable by ether from unacidified- and from acidified-metanilamide urine is mainly acetylmetanilamide (see below). The glucuronide of 4-hydroxymethanilamide has been described in an earlier paper (Williams, 1943) and in the diazo colour reaction it gives molecule for molecule 70–75 % of the intensity of colour shown by metanilamide.

'Total' metanilamide includes all those compounds which can be diazotized and coupled after

Table 3. *Substances reacting with the coupling reagent in the urine from rabbits receiving metanilamide*

Before boiling with acid ('free' metanilamide) Compound	Relative colour intensity*	After boiling with acid ('total' metanilamide) Compound	Relative colour intensity*
Metanilamide	100	Acetylmetanilamide	80
Glucuronide of 4-hydroxymetanilamide	70–75	4-Hydroxymetanilamide	10
Ethereal sulphate of 4-hydroxymetanilamide†	(Probably *c.* 50–70)	Partly hydrolyzed glucuronide of 4-hydroxymetanilamide	Less than 70

* Colour intensity relative to metanilamide.

† Since this ethereal sulphate,  , has not been isolated, its relative colour intensity cannot be given. However, it is likely to give a colour about as intense as the glucuronide. Two compounds containing a strongly acid group *ortho* to an NH_2 group were tested; orthanilic acid,  , gave a relative colour intensity of 50, and *p*-bromoaniline-*o*-sulphonic acid,  , of 59.

acid hydrolysis of metanilamide urine. These include free metanilamide from acetylmetanilamide, 4-hydroxymetanilamide from the ethereal sulphate, and the partly hydrolyzed glucuronide of 4-hydroxymetanilamide. 4-Hydroxymetanilamide gives only one-tenth of the colour given by an equivalent amount of metanilamide (see Table 3).

The difference between 'free' and 'total' metanilamide does not, therefore, give an exact estimate of the amount of acetylated metanilamide. It should in consequence be pointed out that whenever a sulphonamide undergoes appreciable oxidation *in vivo*, measurements of total and free sulphonamide by diazotization and coupling do not give the true amount acetylated. In the case of sulphanilamide (see following paper) the amount of oxidation is small and the difference between free and total sulphanilamide is therefore near to the true value for acetylated sulphanilamide.

The urine of rabbits, which have been given metanilamide orally, is 'normal' in appearance, slightly alkaline, non-reducing to Benedict's reagent, gives no colour with ferric chloride, but gives an intense naphthoresorcinol reaction for glucuronic acid and an intense diazo reaction showing the presence of free aromatic amino groups.

Identification of metabolic products

(*a*) *Isolation of acetylmetanilamide.* 1·5 g. metanilamide (m.p. 142°) were fed in aqueous suspension to a rabbit (2·2 kg.) by stomach tube. The urine was collected during 24 hr., then acidified with dilute HCl and extracted with ether. The ethereal extract was evaporated and the residue, which gave no diazo reaction, was recrystallized (charcoal) twice from hot water. Crystals were obtained which were identified as *m*-acetaminobenzenesulphonamide, m.p. and mixed m.p. 218–219° (Zincke & Müller (1913) give m.p. 217° and Jacobs & Heidelberger (1917) give m.p. 216–219°).

In another experiment 16 g. of metanilamide were fed to three rabbits during 3 days. The urine (2·2 l. including washings) was collected for 4 days. After centrifuging, the urine, without acidification, was extracted continuously for 54 hr. with ether. The ether was evaporated and there remained 6·76 g. of crude acetylmetanilamide (m.p. 175–200°). The product gave only a weak diazo reaction, indicating that it contained only small amounts of unchanged metanilamide. The yield of crude acetyl derivative was equivalent to 34 % of the dose.

The extracted urine was now heated on a water-bath for 2 hr. to remove dissolved ether, and then boiled gently for 2 hr. with 400 ml. conc. HCl. After cooling, the product was made just alkaline to litmus with 40 % (w/v) NaOH and then extracted continuously with ether for 25 hr. On evaporating the ethereal extract, 4·5 g. of crude metanilamide (28 % of dose fed) were obtained which contained small amounts of a phenol which is described later. Thus, by ether extraction of the hydrolyzed and unhydrolyzed 'metanilamide' urine, some 62 % of the material fed can be accounted for.

(*b*) *Isolation of 3-amino-4-hydroxybenzenesulphonamide (4-hydroxymetanilamide).* Ten rabbits were each given 2 g. metanilamide by stomach tube. The urine (1840 ml. including washings) was collected during 2 days. It was centrifuged and gently boiled with 300 ml. conc. HCl for 2 hr., during which time the ethereal sulphate and acetyl derivative, but not the glucuronide, were hydrolyzed. The hydrolyzed mixture was cooled, filtered from some black material and brought to pH 4–5 with solid K_2CO_3. It was then extracted with ether for 50 hr. The extracted urine (MA) was kept for examination later. The ethereal extract (MB) was evaporated leaving a brownish crystalline mass containing some brownish syrupy material. The extract which gave an intense red colour with $FeCl_3$ weighed 13 g. or 65 % of the metanilamide fed.

Chromatographic analysis of extract MB. The extract, MB, was dissolved in 100 ml. hot water, filtered after adding a little charcoal, and cooled. Crystals of metanilamide (5·4 g., m.p. 138°) were filtered off and the filtrate, which gave an intense $FeCl_3$ reaction, was taken to dryness *in vacuo*. The residue was dissolved in 50 ml. absolute ethanol and the solution mixed with 150 ml. dry benzene. The mixture was filtered from a very small amount of extraneous material and then poured on to an alumina column (Savory & Moore's aluminium oxide, standardized according to Brockmann) (7 in. long by 1·5 in. diameter). The column was eluted with 600 ml. 1 : 1 benzene/ethanol, followed by 500 ml. absolute ethanol, until the eluates gave a negative diazo reaction. Up to this point none of the eluates had given a colour with $FeCl_3$, although the column had been washed free of metanilamide. The combined eluates were evaporated and yielded 3·75 g. of metanilamide, m.p. 142–3°, after recrystallization from water.

The alumina column at this point showed three distinct zones, a lower zone devoid of colour, a central yellow zone and an upper light brown zone. The column was, therefore, cut into three parts and each zone, *t* (top), *m* (middle) and *b* (bottom), was extracted with boiling water. Aqueous extracts of zones *m* and *t* were alkaline to litmus. Zone *m* yielded 1·5 g. of solid material after evaporation of the solvent, and zone *t* yielded 0·95 g. of solid. The reaction with $FeCl_3$ of both zones *m* and *t* was purplish red→red→brown. Zone *b* yielded nothing.

The solid from zone *m* was recrystallized (charcoal) from 15 ml. acidulated water and 0·45 g. of slightly brown elongated platelets obtained. The crystals had m.p. 200–201° and mixed m.p. 201–202° with

authentic 3-amino-4-hydroxybenzenesulphonamide, m.p. 202° (Williams, 1943; Kermack, Spragg & Tebrich, 1939). (Found: C, 38·3; H, 4·4; N, 15·3; S, 17·2 %. Calc. for $C_6H_8N_2O_3S$: C, 38·3; H, 4·3; N, 14·9; S, 17·0 %.) They gave with $FeCl_3$ solution a purplish red colour which quickly turned red, then brown with final deposition of a brown precipitate; on diazotization with nitrous acid they gave a deep yellow solution which slowly coupled with ethanolic dimethyl-α-naphthylamine giving a dark-red coloration. These reactions are identical with those given by 3-amino-4-hydroxybenzenesulphonamide (4-hydroxymetanilamide) and establish the identity of the phenol from zone *m*.

The solid from zone *t* also contained the above phenol as indicated by colour reactions although it was not obtained crystalline. The solid extract from zone *t* was dissolved in 10 ml. hot acidulated water (charcoal) and filtered. On cooling no crystals appeared, but on further acidification small colourless plates (0·1 g.), m.p. 119°, separated. These were identified as benzoic acid by a mixed melting-point (119–120°) with authentic benzoic acid. This acid is, of course, derived from hippuric acid which is a normal constituent of rabbit urine.

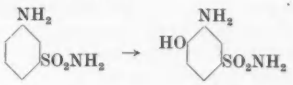
DISCUSSION

Metanilamide undergoes in the rabbit both acetylation and oxidation. Little free metanilamide appears to be excreted, for in two experiments only acetylmetanilamide could be extracted from the urine with ether despite the fact that free metanilamide is easily extractable by ether. The amount of metanilamide acetylated is uncertain. The differences between the 'free' and 'total' metanilamide (apparent acetylation) given in Table 2, yield figures for acetylated metanilamide varying from 18 to 75 % of the dose. The uncertainty of these values has already been discussed. By direct ether extraction of urine for 54 hr. some 34 % of the metanilamide fed can be obtained as acetylmetanilamide, but, on hydrolysis of the extracted urine, a further 28 % of the dose is extractable by ether as free metanilamide. This 28 % must have been derived from unextracted acetylmetanilamide, indicating that up to 62 % of the metanilamide is excreted acetylated. Although these two ways of assessing the amount of metanilamide undergoing acetylation in the body do not give exact results they indicate that metanilamide undergoes appreciable acetylation in the rabbit and that probably about one-half of the dose is excreted acetylated.

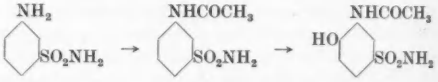
If both the ethereal sulphate and glucuronide outputs of metanilamide-fed rabbits could be estimated, then an exact estimation of the extent of oxidation of metanilamide could be obtained. Only the ethereal sulphate output could be estimated with any degree of accuracy and the sulphate estimations indicated that some 20–25 % of metanilamide is oxidized to a phenol, which is excreted conjugated as an ethereal sulphate. Since the oxidation product of metanilamide is 4-hydroxymetanilamide it is likely that the glucuronide excreted is 4-glucuronosidometanilamide (see Williams, 1943). Such a glucuronide is fairly resistant to acid hydrolysis (cf. Hanson, Mills & Williams, 1944) and its estimation by the naphthoresorcinol-HCl method is unsatisfactory.

4-Hydroxymetanilamide could be formed from metanilamide in three ways, thus:

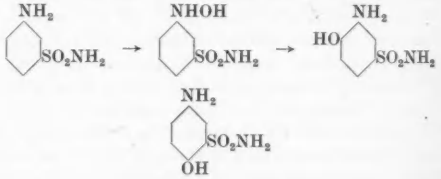
(1) by direct oxidation,



(2) by direct oxidation of the acetyl derivative,



(3) by rearrangement of a hydroxylamine,



I

No evidence was obtained during this work that a hydroxylamine was formed, and if it were formed the presence of a *p*-aminophenol, i.e. 6-hydroxymetanilamide (I), could also be expected. Such a phenol was not detected (the glucuronide of 6-hydroxymetanilamide has been prepared by Williams, 1943).

Regarding mechanisms (1) and (2) it appears that the first is likely to be the correct one. Examination of Table 2 shows that about 30–50 % of the metanilamide fed is excreted in a form which diazotizes and couples with naphthylethylenediamine, yet very little free metanilamide can be extracted from the urine with ether. These facts lead to the conclusion that the 'free' metanilamide estimated by the diazo method is mainly the ethereal sulphate and glucuronide of 4-hydroxymetanilamide in which the amino group is free (see Table 3). This suggests that the ethereal sulphate excreted is that of 4-hydroxymetanilamide and not of 4-hydroxy-3-acetylmetanilamide.

SUMMARY

The fate of metanilamide in the rabbit has been studied.

1. This compound is converted *in vivo* into *m*-acetaminobenzenesulphonamide and the ethereal sulphate and glucuronide of 4-hydroxymetanilamide.

2. About 50 % is acetylated and more than 25 % is oxidized to 4-hydroxymetanilamide.

3. 20–25 % of the metanilamide is excreted as an ethereal sulphate.

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The Metabolism of Sulphonamides

2. THE ISOLATION AND IDENTIFICATION OF 3-HYDROXYSULPHANILAMIDE AS THE OXIDATION PRODUCT OF SULPHANILAMIDE IN THE RABBIT

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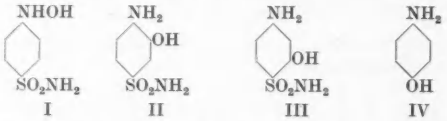
Although it is known that a number of the sulphonamide drugs used in therapy undergo oxidation *in vivo*, none of the oxidation products have hitherto been chemically identified. Scudi (1940) isolated from the urine of dogs receiving sulphapyridine a monohydroxysulphapyridine, m.p. 180–181°, but left its structure undetermined. Weber, Lalich & Major (1943) isolated from dog's urine a glucuronide of a hydroxysulphapyridine in which the hydroxyl group is attached to the pyridine ring. No other data of this type has been published on sulphonamide oxidation products.

The determination of the exact chemical nature and the biological properties of sulphonamide oxidation products is highly important since certain theories relative to the mode of action of sulphonamide drugs involve the formation of oxidation products. Mayer & Oechslin (1937) suggested that the activity of sulphanilamide was due to its conversion *in vivo* to an oxidation product which they suggested might be *p*-hydroxylaminobenzenesulphonamide (N^4-hydroxysulphanilamide), this being the effective therapeutic agent. Another theory involving the hydroxylamine derivative was put forward by Locke, Main & Mellon (1938), who assumed that since hydroxylamine (NH_2OH) and oximes inhibit catalase, *p*-hydroxylaminobenzenesulphonamide would do the same (this is actually true; see Sevag, Shelburne & Ibsen (1942)). Hydrogen peroxide, normally resulting from the metabolic activities of bacteria such as streptococci and pneumococci, would not be removed by catalase if *p*-hydroxylaminobenzenesulphonamide were present and would therefore accumulate, and in turn destroy the bacteria. Some workers (e.g. Rosenthal & Bauer, 1939; James, 1940) claimed to have detected hydroxylamine derivatives in the urine of patients and of animals receiving sulphanilamide, but these claims were shown to be invalid by Thorpe, Williams & Shelswell (1941). In all, some four compounds have been suggested as oxidation products of sulphanilamide, i.e. N^4-hydroxy- (I), 3-hydroxy- (II) and 2-hydroxysulphanilamide (III) and *p*-aminophenol (IV) (cf. James, 1940; Thorpe *et al.*

1941), but hitherto, unequivocal proof has not been provided concerning the precise chemical nature of the oxidation product or products.



It has also been suggested (e.g. Ottenberg & Fox, 1938) that cyanosis produced in patients by sulphanilamide may be due to oxidation products.

METHODS

Ethereal sulphate and free and total sulphanilamide excreted by rabbits were determined as described by Dobson & Williams (1946).

RESULTS

Quantitative determinations

Ethereal sulphate excretion. In an earlier paper (Shelswell & Williams, 1940) it was shown that rabbits excreted from 6 to 12 % of doses of sulphanilamide varying from 0·2 to 1·0 g./kg. as an ethereal sulphate. In the present work, determinations confirmed these results. Two rabbits were given 500 mg./kg. orally; one excreted in 2 days 9·5 and the other 7·2 % of the dose as ethereal sulphate.

Free and total sulphanilamide. The results are given in Table 1.

Table 1. *Excretion of free and total sulphanilamide by the rabbit*

Rabbit no.	Dose (mg./kg.)	Time of excretion (days)	Sulphanilamide excreted, % of dose		
			Free	Total	Acetylated
2	100	2	54	94	40
5	100	2	8	90	81
8	100	2	32	89	57
2	250	2	45	57	12
2	250	1	52	63	11
4	250	1	62	81	19
5	250	1	15	44	29

It has been reported by Marshall, Emerson & Cutting (1937) and Gilligan (1945) among others, that acetylation of the sulphonamide drugs is an extremely variable process both in man and animals. The results in Table 1 add further confirmation to these reports.

Identification of metabolic products

The isolation of 3-hydroxysulphanilamide. Several unsuccessful attempts were made to isolate 3-hydroxysulphanilamide in a pure crystalline state from urines in which its presence was detected by colour reactions. The following is a description of an experiment in which the phenol was isolated in a crystalline state.

Twenty rabbits (2–3 kg. wt.) were each given by stomach tube 2 g. of sulphanilamide suspended in water. The dose was repeated on the two following days until a total of 120 g. of sulphanilamide had been fed. The urine was collected for 5 days after the first dose, the total volume (including funnel washings) being 10 l. It was alkaline to litmus.

(*a*) *Examination of the urinary deposits for azoxybenzene-4:4′-disulphonamide.* It is known that *p*-hydroxylaminobenzenesulphonamide is rapidly transformed in neutral or alkaline solution into azoxybenzene-4:4′-disulphonamide (Bratton, White & Marshall, 1939). If any of the hydroxylamine compound were excreted it would be rapidly transformed in the alkaline urine of the rabbit into the azoxy compound. The latter is very insoluble and would, therefore, be found in the urinary deposit together with acetylsulphanilamide. A separate experiment had shown that pure *p*-hydroxylaminobenzenesulphonamide, dissolved in normal rabbit urine (alkaline to litmus), was rapidly precipitated as insoluble azoxybenzenedisulphonamide. The latter compound can easily be detected in urinary deposits by means of its characteristic ultra-violet absorption spectrum, which in ethanol or in KOH shows a large band with maximum absorption at 333 mμ. (see Fig. 1).

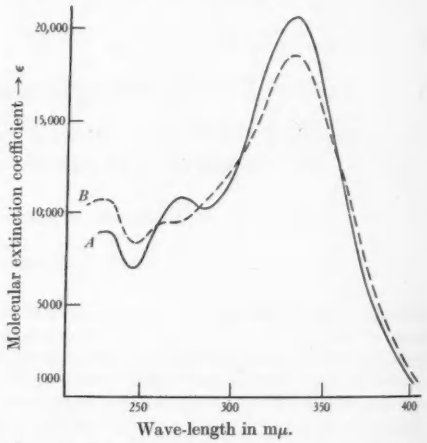


Fig. 1. Spectral absorption of azoxybenzene-4:4′-disulphonamide. *A*. In ethanol: $\lambda_{max.}$ 333 mμ., $\epsilon_{max.}$ 20,665. *B*. In 0·01 N-KOH: $\lambda_{max.}$ 333 mμ., $\epsilon_{max.}$, 18,666.

The urine collected during the first 2 days of the experiment was centrifuged and the deposit obtained was extracted with 350 ml. 10% (w/v) NaOH for 3 days. The mixture was filtered and the filtrate examined spectrographically for azoxybenzene-4:4′-disulphonamide. The result was negative. On acidifying a little of the alkaline extracted with strong HCl and allowing it to stand, long needles of acetylsulphanilamide, m.p. 216°, separated.

The urine collected on the third day was similarly treated, but spectroscopic examination failed to reveal any trace of the azoxy compound in the deposit.

The centrifuged urine of the first 2 days was now allowed to stand at 0° for 6 days and the deposit which formed

during this time was collected on the centrifuge. Spectroscopic examination of this deposit again gave a negative result for the azoxy compound.

(*b*) *Hydrolysis and extraction of the urine.* The centrifuged urine (*c.* 10 l.) was concentrated *in vacuo* to 675 ml. To this 400 ml. conc. HCl were added and the mixture was boiled for 2 hr. To the cooled mixture 40% (w/v) NaOH was added until it was just acid to litmus. The mixture was filtered from some black material (this material was examined for the azoxy compound with negative results), and kept overnight at 0°. A deposit of crude sulphanilamide (30 g.), which contained no azoxybenzenedisulphonamide, was removed by filtration.

The urine was now extracted with diethyl ether in a continuous extractor for 40 hr. Evaporation of the ether left 54·3 g. of pale brown crystalline material. This was dissolved in 400 ml. hot water and charcoal added. The solution was filtered and allowed to crystallize at 0°. 30 g. of sulphanilamide, m.p. 163–164° (free from azoxybenzenedisulphonamide by spectroscopic test), were filtered off. The filtrate gave a strong $FeCl_3$ colour reaction for 3-hydroxysulphanilamide, i.e. purplish red to red to brown. It was taken to dryness *in vacuo* leaving a semi-solid pale brown mass (D).

(*c*) *Chromatographic analysis of D.* The material *D* was dissolved in 200 ml. absolute ethanol and then 200 ml. benzene were added. The solution was now chromatographed on an alumina column (aluminium oxide standardized according to Brockmann, Savory and Moore, Ltd., London). The column was washed with ethanol until the diazo test (nitrous acid and dimethyl-α-naphthylamine) carried out on the washings was very feeble. On removal of the solvent the washings yielded 6·15 g. of crystalline material, which was identified as mainly sulphanilamide.

The 3-hydroxysulphanilamide remained on the column which showed a broad yellow band (*B*), located near the bottom, the rest (*T*) of the column above this band having a pale brownish yellow colour. Colour tests indicated that the main bulk of the phenol was in the broad yellow band *B* with smaller quantities in *T*. The column was cut, separating the two parts. *B* was extracted with hot water until it no longer gave a colour with $FeCl_3$. The extract was filtered and taken to dryness *in vacuo*, affording 1·7 g. of a pale brown, sticky solid giving intense colour reactions for 3-hydroxysulphanilamide. This solid was recrystallized from a little hot water with addition of a little charcoal, and after several days there were obtained 200 mg. of crude 3-hydroxysulphanilamide, m.p. 148°. On recrystallization from hot water, it formed small prisms, m.p. 161°, mixed m.p. 161° with authentic 3-hydroxysulphanilamide, m.p. 164°. (Found: C, 38·7; H, 4·4; N, 15·2; S, 17·2%. Calc. for $C_6H_8N_2SO_3$: C, 38·3; H, 4·3; N, 14·9; S, 17·0%.) The crystals gave all the colour reactions of authentic 3-hydroxysulphanilamide, which are as follows: (*a*) On adding $NaNO_2$ and HCl to a solution of the phenol an intense yellow colour is obtained. If ethanolic dimethyl-α-naphthylamine is added to the diazotized solution (excess nitrous acid having been destroyed by ammonium sulphamate) coupling to an orange-red colour takes place slowly. (*b*) On adding 2% $FeCl_3$ to the phenol a purplish red colour which rapidly turns red and finally brown, is obtained. If this reaction is carried out in the presence of peroxide-free ether, the ether becomes coloured an intense ruby red. This ruby red ethereal solution shows an absorption band with a maximum at 530 mμ. (this reaction will be described in greater detail in a later publication) and the colour is stable for about a day. (*c*) On heating an ethanolic solution of the phenol with 2:4-dinitrobenzaldehyde in the presence of a few drops of glacial acetic acid for about 15 min., a mixture is obtained which gives an intense red colour with alkali (10% KOH). The red colour is discharged by acid.

The crystals were also carefully compared with all the other possible oxidation products of sulphanilamide (see introduction). The above tests and analyses provide unequivocal proof of the identity of the phenol isolated.

The portion *T* of the column was treated in a similar manner to *B* and from it there was obtained 1·3 g. of a sticky solid which yielded, on repeated recrystallization from water, 55 mg. of 3-hydroxysulphanilamide, m.p. 160°, mixed m.p. 163° with authentic phenol, m.p. 164°.

The injection of p-hydroxylaminobenzenesulphonamide. Water was heated to boiling and allowed to cool to 40–45° with a stream of nitrogen passing through it. 250 mg. of *p*-hydroxylaminobenzenesulphonamide (m.p. 140°) were dissolved in 15 ml. of this water and 10 ml. of the solution were quickly injected subcutaneously into a rabbit. The animal became cyanosed after about an hour. Its urine (60 ml.) was collected for 19 hr., centrifuged and the deposit tested for azoxybenzene-4:4′-disulphonamide. The result was negative.

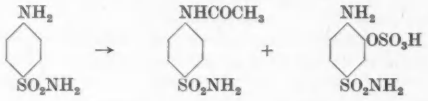
The experiment was repeated with two rabbits each receiving 200 mg. of the hydroxylamine; both animals became cyanosed. The urine was collected separately on each of the following 2 days. The two deposits were examined for the azoxy compound but only uncertain traces were found spectroscopically.

The combined urines were extracted for 4 hr. with peroxide-free ether, which on evaporation left a brownish crystalline residue. Qualitative tests showed that this residue contained free sulphanilamide but no hydroxylamine derivative. On recrystallization from water the residue yielded 50 mg. of pure acetylsulphanilamide (m.p. 213°).

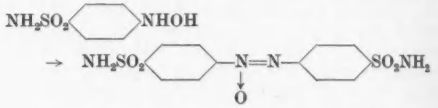
DISCUSSION

The present work proves that the oxidation product excreted in the urine of rabbits fed sulphanilamide is 3-hydroxy-4-aminobenzenesulphonamide (3-hydroxysulphanilamide). No evidence was obtained of the presence of other oxidation products such as *p*-hydroxylaminobenzenesulphonamide and its *N*-acetyl derivative, 2-hydroxysulphanilamide and *p*-aminophenol. Other work in progress in this laboratory has shown that 3-hydroxysulphanilamide also occurs in the urine of hospital patients treated with sulphanilamide. The phenol is not excreted as such but appears in the urine mainly as an ethereal sulphate. Whether or not a glucuronide is excreted after sulphanilamide administration appears to be doubtful. Gilligan (1945) has reported that sulphanilamide does not increase the glucuronic acid output in man and Scudi & Robinson (1941) found a similar result with rats. It appears likely that, since the amount of 3-hydroxysulphanilamide formed is small, not enough is produced to give rise to appreciable glucuronide formation, the sulphate

mechanism being able to cope with most of the phenol. When, however, 3-hydroxysulphanilamide is fed to rabbits, it is partly excreted in conjugation with glucuronic acid (Williams, 1943). It may be stated, therefore, that when sulphanilamide is fed to rabbits, the urine is found to contain free sulphanilamide, acetylsulphanilamide and the ethereal sulphate of 3-hydroxysulphanilamide,



Concerning the formation of *p*-hydroxylaminobenzenesulphonamide, the present work does not entirely eliminate the possibility that it may be formed *in vivo*, but it does prove that this hydroxylamine is not excreted in the urine. The possibility still exists that *p*-hydroxylaminobenzenesulphonamide may be formed and rearranged *in vivo* to 3-hydroxysulphanilamide. There is, however, circumstantial evidence against this possibility. First, Bratton *et al.* (1939) have shown that on injection of this hydroxylamine into dogs, it is largely converted into sulphanilamide (dogs do not acetylate the aromatic amino group). In the rabbit, the subcutaneously injected hydroxylamine is largely converted into sulphanilamide and acetylsulphanilamide. Secondly, in the analogous case of *p*-hydroxybenzenesulphonamide, Williams (1941) has proved that this compound is oxidized in the rabbit to catechol-4-sulphonamide, a result which shows that the benzene ring can be oxidized in the *meta* position to a sulphonamide group and *ortho* to a hydroxyl group when both these groups occur together *para* to each other. Thirdly, the chemical transformations of *p*-hydroxylaminobenzenesulphonamide show that this compound is transformed in neutral or alkaline solution solely to azoxybenzene-4:4′-disulphonamide (Bratton *et al.* 1939; Burton & Walker, 1943),



whereas on heating in acid solution it forms the azoxy compound and *p*-aminophenol (Burton & Walker, 1943). *p*-Aminophenol was not detected and this confirms earlier work (Thorpe *et al.* 1941). It has not yet been shown that this hydroxylamine can be converted chemically into 3-hydroxysulphanilamide.

SUMMARY

1. The oxidation product of sulphanilamide excreted by rabbits has been isolated and proved to be 3-hydroxy-4-aminobenzenesulphonamide. This compound is excreted as an ethereal sulphate.

2. Injection of *p*-hydroxylaminobenzenesulphonamide into rabbits results in the excretion of sulphanilamide and acetylsulphanilamide.

3. The ultra-violet absorption spectrum of azoxybenzene-4:4′-disulphonamide has been recorded.

The author wishes to thank Prof. R. A. Morton for his invaluable assistance with the spectroscopic data quoted in this paper.

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Studies in Detoxication

14. THE METABOLISM OF VERATRALDEHYDE AND VERATRIC ACID IN THE RABBIT

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The work described in this paper had two objectives. First, it was hoped that a more stable aroylglucuronide (veratroylglucuronide and anisoylglucuronide) than benzoylglucuronide (Quick, 1926; Pryde & Williams, 1933; Goebel, 1938) could be prepared for constitutional studies. In the second place, it was considered possible that a comparative study of the metabolism of veratric acid (3:4-dimethoxybenzoic acid) and its aldehyde might throw light on the mode of oxidation of aromatic aldehydes to the corresponding acids, since early experiments had shown that the yields of crystalline veratroylglucuronide from rabbit urine were consistently higher when veratraldehyde was fed than when veratric acid was fed.

No previous study on the fate of veratraldehyde or of anisaldehyde has been recorded. Marfori (1897) states that veratric acid is excreted unchanged; our work will show this statement is only partly true.

METHODS AND RESULTS

Veratraldehyde (m.p. 46°) was prepared by the Reimer-Tiemann reaction (Johnson & Stevenson, 1936); veratric acid (m.p. 181°) was prepared in a similar manner (Kostanecki & Tambor, 1906). For feeding, the aldehyde was melted in warm water and the acid was neutralized with Na_2CO_3 solution.

Ethereal sulphate excretion. This was determined by the Folin gravimetric method (Williams, 1938). It was assumed that the increase in ethereal sulphate was due to conjugated catechol, 1 mol. of the phenol being combined with 1 or 2 of sulphuric acid. Hence in calculating the veratric aldehyde or acid equivalent to the ethereal sulphate increase, it was assumed that 1 or 2 mol. of $SO_3 \equiv 1$ mol. of veratric aldehyde or acid.

Total and free veratric acid excretion. The method used was identical with that of Sammons & Williams (1941) for the estimation of free and total vanillic

Table 1. *The distribution of metabolites in the urine of rabbits after the administration of veratric aldehyde and veratric acid*

(Urines for analysis were collected for 2 days after dosage.)

Substance fed	Rabbit no.	Dose (g.)	Free veratric acid (g.)	Free veratric acid (% of dose)	Total veratric acid (g.)	Total veratric acid (% of dose)	Veratroyl-glucuronide* % of dose	mg. SO_3	Ethereal sulphate excretion calculated as: Mono-sulphate (% of dose)	Ethereal sulphate excretion calculated as: Disul-phate (% of dose)	Total veratraldehyde or veratric acid excreted (% of dose): Including mono-sulphate	Total veratraldehyde or veratric acid excreted (% of dose): Including disul-phate
Veratral-dehyde	71	2·2	0·790	35·7	1·440	64·9	29·2	75	7·0	3·5	71·9	68·4
	76	2·2	0·261	10·8	1·462	60·6	49·8	48	4·4	2·2	65·0	62·8
	77	1·9	0·503	24·2	1·348	64·7	40·5	50	5·2	2·6	69·9	67·3
	78	2·2	0·983	40·8	1·500	62·2	21·4	43	4·0	2·0	66·2	64·2
	78	2·2	0·318	13·2	1·564	64·8	51·6	68	6·2	3·1	71·0	67·9
	79	1·9	0·848	40·7	1·641	78·7	38·0	64	6·6	3·3	85·3	82·0
Average				(27·6)		66·0	(38·4)		5·6	2·8	71·6	68·8
Veratric acid	76	2·0	0·750	37·4	1·390	69·5	32·0	58	5·8	2·9	75·3	72·4
	77	1·9	0·725	38·1	1·290	67·9	29·8	42	4·4	2·2	72·3	70·1
	78	2·25	0·897	39·9	1·579	70·2	30·3	25	2·2	1·1	72·4	71·3
	79	2·0	0·984	49·2	1·417	70·8	21·6	41	4·0	2·0	74·8	72·8
Average				41·2		69·6	28·4		4·2	2·1	75·8	71·6

* I.e. difference between excreted total and free veratric acid.

acid in urine, i.e. ether extraction and a methoxyl estimation. The method was found to be reliable for total veratric acid, but the amount of free veratric acid was uncertain owing to the instability of veratroylglucuronide in urine. The results are given in Table 1.

Isolation of veratroylglucuronide

(*a*) *From veratraldehyde.* 2 g. of veratraldehyde were administered by stomach tube to each of ten rabbits and the urine (1620 ml.) collected during the following 24 hr. All reagents were ice-cold and were stored at 0°. The urine, acidified with a few drops of glacial acetic acid, was treated with 400 ml. of saturated normal lead acetate and centrifuged. The precipitate was discarded and the clear supernatant liquor made faintly alkaline with a few drops of strong ammonia. Saturated basic lead acetate (500 ml.) was now added and the whole centrifuged. The precipitate was washed twice on the centrifuge with ice-cold water and made into a thin cream with water. The lead was removed with H_2S and the filtrate aerated. *Veratroylglucuronide* crystallized overnight at 0°. A further quantity was obtained on concentrating the mother liquor *in vacuo* at 30–35° (total yield, 11 g.). On recrystallization from hot water, it formed needles, m.p. 169–170°, showing $[\alpha]_D^{23°} - 13{\cdot}1°$ ($c = 0{\cdot}9$ in water) and $[\alpha]_D^{24°} - 2{\cdot}5°$ ($c = 1$ in methanol). From methanol the glucuronide separated as a gelatinous mass which crystallized only when water was added. It reduced Benedict's and Fehling's solution and was soluble in methanol, acetone and methylethylketone, sparingly soluble in ethyl acetate and cold water and insoluble in ether. (Found: C, 45·9; H, 5·8; OMe, 15·9; H_2O, 8·8 %; equiv., 393, after hydrolysis, 195. $C_{15}H_{18}O_{10}.2H_2O$ requires C, 45·7; H, 5·6; OMe, 15·7; H_2O, 9·1 %; equiv., 394, after hydrolysis 197.)

(*b*) *From veratric acid.* A rabbit was given 2 g. veratric acid dissolved in dilute Na_2CO_3. The procedure was the same as described in the preceding paragraph and 0·5 g. of veratroylglucuronide was isolated, m.p. 169° after recrystallization. (Found: H_2O, 8·9 %. Calc. for $2H_2O$, 9·1 %.)

The action of NaCN *on veratroylglucuronide*

Quick (1926; cf. Pryde & Williams, 1933) showed that when benzoylglucuronide was treated with NaCN, it underwent a change in optical rotation, for which there is no complete explanation. Goebel (1938) suggests that the phenomenon is the result of a complex series of reactions initiated by a migration of the benzoyl group, catalyzed by OH ions, from the aldehydic to one of the remaining carbon atoms of glucuronic acid. The following experiments show that veratroylglucuronide undergoes a similar rotatory change. On treatment of a 1 % solution of the glucuronide with 1·5 molecular equivalents of NaCN, the rotation of the solution changed from $[\alpha]_D^{23°} - 13{\cdot}4°$ to $+1°$ in 110 min. With increase of the amount of NaCN to 2 mol., the change was less rapid, $[\alpha]_D^{24°} - 15{\cdot}8°$ to $-7{\cdot}1°$ in 6·5 hr. and similar results were observed with 1 or 5 % solutions of the glucuronide. When the cyanide-treated solution had reached a constant rotation, it was no longer reducing to Benedict's or Fehling's solution.

1 g. of the glucuronide (1 mol.) and 0·2487 g. of NaCN (2 mol.) were dissolved in 20 ml. water. When the solution had attained a constant rotation (30 hr.) it was acidified with 2 ml. of 2N-HCl and then extracted with ether. Removal of the ether left 0·156 g. (37·5 % theory) of veratric acid, m.p. and mixed m.p. 179°, after recrystallization. The solution which remained after ether extraction was made slightly acid to congo-red and then taken to a syrup which was non-reducing. No positive result concerning the identity of this syrup was obtained.

Veratroylglucuronide methyl ester

This was prepared in three ways (diazomethane, methyl alcoholic HCl and methyl iodide plus silver oxide in the cold) and each method gave the same product. Only one method need be described here. Anhydrous veratroylglucuronide (2·7 g.) was dissolved in dry methanol (25 ml.) by heating under reflux. The solution was cooled to $-5°$ and treated with diazomethane in the usual manner. After 2 hr. at 0°, the solution was concentrated at 35° to a gelatinous solid which was purified by precipitation from methanol with ether. The *ester* had m.p. 167° and $[\alpha]_D^{18°} - 9{\cdot}9°$ ($c = 1$ in water). It reduced Fehling's and Benedict's reagents and gave a bright yellow colour with NaOH on standing or warming. (Found: OMe, 21 %. $C_{16}H_{20}O_{10}$ requires OMe, 25·0 %.)

This methyl ester (1 g.) was now dissolved in pyridine (10 ml.) and acetic anhydride (6 ml.) and allowed to stand overnight. The solution was then poured into water and the white crystalline precipitate (0·5 g.) was collected and recrystallized from aqueous methanol. The *triacetylveratroylglucuronide methyl ester* formed large needles, m.p. 144·5° and $[\alpha]_D^{24°} - 31{\cdot}3°$ ($c = 0{\cdot}9$ in methanol) and was reducing. (Found: C, 53·2; H, 5·5; OMe, 18·2 %. $C_{22}H_{28}O_{13}$ requires C, 53·0; H, 5·3; OMe, 18·7 %.)

Complete methylation of the methyl ester. Several attempts were made to methylate veratroylglucuronide with methyl iodide and silver oxide. The product obtained was a pale yellow syrup, slightly acidic to congo-red paper and reducing to Benedict's reagent. The methoxyl content was 41·5 %; fully methylated veratroylglucuronide requires 44·9 %. This product was not homogeneous and could be fractionated by means of light petroleum and ether mixtures. Although much time was spent (see Sammons, 1942) in investigating the nature of this syrup, the only definite hydrolytic product

was veratric acid. The results indicated that veratroylglucuronide was not stable during methylation, although the veratroyl group was not removed during the process.

Hydrolysis of veratroylglucuronide by normal rabbit urine

Veratroylglucuronide was dissolved in normal rabbit urine (alkaline) and the free veratric acid content was determined at intervals. The results for the urine of two different rabbits are given in Table 2.

Table 2. *The decomposition of veratroylglucuronide in rabbit urine*

Vol. of urine (ml.)	Glucuronide added (g.)	Glucuronide hydrolyzed (%) in (hr.) 0	2	24	48
50	0·43	—	17·9	26·5	83·1
50	0·16	9·7	—	85·6	—

From these urines, crystalline veratric acid, identical with an authentic sample, was isolated by extraction with ether.

Isolation of free veratric acid from veratraldehyde urine

A rabbit was given 2 g. of the aldehyde and the urine (90 ml.) collected for 24 hr. Sulphuric acid (9 ml. of 10 %) was added to the urine, which was then extracted with ether for 4 hr. Evaporation of the ether gave a semi-crystalline residue which was now twice extracted with hot toluene. Veratric acid (0·26 g. or 12 % of the dose fed, m.p. and mixed m.p. 181° after recrystallization from water) was obtained on evaporation of the toluene extract. The residue after extraction with toluene was tested for vanillic acid by the colour reaction of Thorpe & Williams (1937) with uniformly negative results. No evidence was obtained for the presence in this residue of veratroylglycine, although if a glycine derivative were formed it would be expected to occur here.

Examination of the urine for free veratraldehyde

Urine from rabbits fed with veratraldehyde (1 g./kg.) was collected periodically (2, 4 and 24 hr. after feeding). All urines gave negative tests for veratraldehyde. The tests used were the colour reaction of Sammons & Williams (1941) for *p*-methoxybenzaldehydes and the use of 2:4-dinitrophenylhydrazine. No unchanged aldehyde group was detected although free and conjugated veratric acid was being excreted 4 hr. after feeding the aldehyde.

Examination of the urine for catechol and protocatechuic acid

After the administration of either veratraldehyde or veratric acid the urine gave a green colour (red in alkali) in neutral solution with $FeCl_3$ after acid hydrolysis. No reaction was obtained with unhydrolyzed urines. This indicated that the urines contained either catechol or protocatechuic acid or both. Both these substances are possible metabolic products of veratraldehyde. Preusse (1878–9) has devised a method whereby catechol and protocatechuic acid can be detected in the presence of each other in biological material such as urine. The principle of the method is that whereas both catechol and protocatechuic acid can be extracted by ether from acid solutions, only catechol can be extracted from alkaline (Na_2CO_3) solutions. 50 ml. of 'veratraldehyde urine' were made alkaline with Na_2CO_3 and extracted in a continuous extractor with ether. The other was evaporated and the residue dissolved in water. This solution gave a green colour with $FeCl_3$ indicating the presence of free catechol. The alkaline urine was now acidified and re-extracted with ether. The residue from the ether extract gave no colour with $FeCl_3$ showing that no free protocatechuic acid was present. The acidified urine, thus twice extracted, was now boiled to hydrolyze conjugated compounds and again extracted with ether when alkaline and acid. The alkaline but not the acid extract again gave a green colour with $FeCl_3$, thus indicating the presence of conjugated catechol but not of conjugated protocatechuic acid. Similar, but much more striking results were obtained on the filtrate from veratroylglucuronide as obtained from the basic lead acetate precipitate above.

Isolation of tetrabromocatechol. The basic lead precipitate from rabbit urine after veratraldehyde feeding was treated with H_2S. The PbS was filtered off and the veratroylglucuronide isolated as described above. The mother liquors from the preparation of the glucuronide were made alkaline with Na_2CO_3 and continuously extracted with ether. The extract (*a*) contained catechol. The solution was now made acid and extracted (*b*) but no colour was obtained with $FeCl_3$. The acid solution was now boiled for an hour, made alkaline by Na_2CO_3 and extracted with ether. The extract (*c*) contained catechol. On acidification and further extraction, nothing which reacted with $FeCl_3$ was found. The ether extracts (*a*) and (*c*) were evaporated to a reddish brown syrup. Attempts to sublime the catechol from the syrup were not successful. The syrup was therefore extracted with CCl_4 and the extract treated with a few drops of bromine. On standing overnight yellowish needles (50 mg.) separated. These were collected and twice recrystallized

from methanol containing a little water. Finally, 15 mg. of slightly coloured tetrabromocatechol were obtained. They gave a dark blue colour with $FeCl_3$ and had m.p. 188° which was not depressed by an authentic sample, m.p. 192°. (Found: Br, 70·3 %. Calc. for $C_6H_2O_2Br_4$: Br, 75·1 %.) Stenhouse (1875) gives m.p. 187° and Frejka & Sefránek (1935) give m.p. 191–192°.

Anisoylglucuronide

2 g. of anisaldehyde (*p*-methoxybenzaldehyde, b.p. 248°) were fed to each of six rabbits. The urine was collected for 24 hr. and the procedure for the isolation of veratroylglucuronide followed exactly. *Anisoylglucuronide* was obtained as a non-crystalline gelatinous mass which dried to an amorphous white powder (yield, 1·5 g.). It could not be crystallized from any solvent tried. It was acidic and laevorotatory; it reduced Benedict's reagent and gave an intense naphthoresorcinol reaction.

Triacetylanisoylglucuronide methyl ester. Dry anisoylglucuronide (0·5 g.) was converted to an amorphous *anisoylglucuronide methyl ester* with methanol saturated with gaseous HCl at 0°. The ester (0·4 g.) was acetylated as described for the veratroyl derivative. *Triacetylanisoylglucuronide methyl ester* (0·4 g.) was isolated as large needles (from aqueous methanol), m.p. 158° and $[\alpha]_D^{21°} = -20·5°$ ($c = 1·2$ in chloroform). It was not sufficiently soluble in methanol to give a 1 % solution. (Found: C, 53·9; H, 5·3; OMe, 13·5 %. $C_{21}H_{25}O_{12}$ requires C, 53·8; H, 5·2; OMe, 13·2 %.)

DISCUSSION

Approximately 70 % of the veratric acid and aldehyde fed to rabbits can be accounted for as metabolites excreted in the urine. The main metabolites in each case are veratric acid and veratroylglucuronide. The relative amounts of these metabolites excreted, however, are uncertain, for, whereas the estimations of total veratric acid excreted are reliable, those of free veratric acid and consequently of veratroylglucuronide are unreliable (see Table 1). The reason for this became clear when a study of the stability of veratroylglucuronide in normal rabbit urine was carried out. This compound decomposes fairly rapidly in rabbit urine (Table 2), presumably owing to the alkalinity of the latter. The figures in Table 1 do suggest, however, that the excretion of veratroylglucuronide is greater after feeding the aldehyde than after the acid. This suggestion receives some support from the observation that the yields of the crystalline glucuronide were always greater when the aldehyde was fed than when the acid was fed. But we feel that our results do not allow us to make a decision on this point.

If it were true that the glucuronide output from the aldehyde was greater than from the acid, then the possibility arises that excreted veratroylglucuronide is not formed from veratraldehyde only via veratric acid thus:

$$R.CHO \rightarrow R.COOH \rightarrow R.CO.OC_6H_9O_6,$$

where $R = CH_3O$, CH_3O (substituted benzene ring)

An alternative possibility is that the intermediates are the aldehyde hydrate and its glucuronide, the latter being converted to veratroylglucuronide by a dehydrogenation thus:

$$R.CHO \rightarrow R.CH(OH)_2 \rightarrow R.CH(OH)(OC_6H_9O_6) \xrightarrow{-2H} R.CO.OC_6H_9O_6.$$

The notion that an aldehyde may be oxidized, chemically and biologically, to an acid via the aldehyde hydrate is well known (cf. Wieland, 1932).

A similar possibility arises when aldehydes are converted to the glucuronides of the corresponding alcohols thus:

$$R'.CHO \rightarrow R'.CH(OH)_2 \rightarrow R'.CH(OH)(OC_6H_9O_6) \xrightarrow{-O} R'.CH_2OC_6H_9O_6.$$

This latter suggestion receives some support from the work of Lehmann & Knoefel (1938) on the pharmacodynamics of chloral hydrate and trichloroethanol. It is generally believed that chloral hydrate is detoxicated by being reduced to trichloroethanol, which then conjugates to form trichloroethylglucuronide (urochloralic acid). Lehmann & Knoefel (1938) found that the duration of the hypnotic action of chloral hydrate is briefer than that of trichloroethanol and that the conversion of the latter to inactive metabolites proceeds very slowly. They further state that 'if chloral hydrate were reduced in the organism to trichloroethanol, the duration of action of the former should be longer, as an extra step is required for its detoxication. Since the effect of chloral hydrate is briefer, it seems unlikely that it forms trichloroethanol as such'.

It is, therefore, obvious that there may be some doubt about the mechanism of the transformation of aldehydes to the glucuronides of the corresponding acids or alcohols, and further elucidation is necessary.

The possibility of the formation of veratroylglucuronide from the aldehyde via the acid, however, cannot be entirely eliminated, since the acid itself forms the glucuronide. This process (with the aldehyde hydrate as an intermediate) could be represented as follows:

$$R.CHO \rightarrow R.CH(OH)_2 \rightarrow R.COOH \rightarrow R.CO.OC_6H_9O_6.$$

Catechol is also a metabolite of both veratric aldehyde and acid. Its formation from veratraldehyde involves oxidation, decarboxylation and de-

methylation. It appears in the urine in a combined form which could be a di- or a mono-ethereal sulphate (cf. adrenaline; Richter, 1940; Deichmann, 1943).

The preparation of anisoylglucuronide has not been described before, but Quick (1932) has reported that the corresponding acid, anisic acid, gives rise in the dog and in man to a glucuronide which was not isolated and to a glycine conjugate, *p*-methoxyhippuric acid, which was isolated.

SUMMARY

The fate of veratraldehyde of veratric acid and of anisaldehyde in the rabbit has been studied:

(1) In the case of veratraldehyde, about 70 % of the dose can be accounted for as veratric acid (*c.* 28 %) and veratroylglucuronide (*c.* 38 %). There is some uncertainty regarding the relative amounts of these two metabolites owing to the instability of the glucuronide in rabbit urine.

(2) About 70 % of veratric acid can also be accounted for, about 41 % as free veratric acid and about 28 % as its glucuronide.

(3) The results suggest that the amount of glucuronide formed from the aldehyde is greater than from the acid. The implications of this suggestion are discussed.

(4) A minor metabolite of both veratric acid and aldehyde was identified as catechol, which was responsible for the small increase in ethereal sulphate output. Vanillic acid derivatives, veratroylglycine and protocatechuic acid were not found in the urine.

(5) Veratroylglucuronide and some of its derivatives have been prepared and some of their properties described.

(6) Anisoylglucuronide has been prepared from the urine of anisaldehyde-fed rabbits and some of its derivatives have been described.

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The Esterification of Glycerol with Hippuric Acid and the Behaviour of the Resulting Product towards Enzymes

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The present paper is a report on the first of a series of ester compounds of glycerol with amino-acid derivatives which are being synthesized in order to investigate their chemical properties and behaviour towards enzymes. The synthesis of esters of the amino-acids with glycerol has been twice reported. Abderhalden & Guggenheim (1910) obtained the ester of tyrosine with glycerol and later Fodor & Weizmann (1926) and Weizmann, Haskelberg & Malkova (1929) (cf. Haskelberg, 1930) prepared the mono-ester of glycerol with leucine. Though other esters of glycerol with amino-acids (alanine and glycine esters) were prepared by Fodor & Weizmann (1910) crystallization of these compounds presented great difficulty and in the case of the alanine and glycine esters was unsuccessful.

This paper describes an attempt to obtain a crystalline product from glycerol and glycine by first substituting the free amino group of glycine with the benzoyl radical as in hippuric acid. The behaviour of the resulting ester towards lipases and esterases was investigated, but this could not be done in the case of the leucine ester owing to its extreme instability in aqueous solution. Preliminary work on the esters of glutamic acid and

pyrollidone carboxylic acid with glycerol has shown that the former did not crystallize, although a saponifiable fraction was obtained, whereas the latter gave a crystalline product.

EXPERIMENTAL

(With the collaboration of Miss S. Bermann)

Preparation of hippurylglyceride

Whereas Fodor & Weizmann (1926) reported a strongly exothermic reaction when the sodium salts of the amino-acids employed were mixed with glycerol chlorohydrin by grinding in a mortar, this was not observed with the sodium salt of hippuric acid and glycerol chlorohydrin. Higher temperatures were therefore employed.

After thorough mixing of 30 g. of the sodium salt of hippuric acid with an equivalent amount of glycerol chlorohydrin in a porcelain mortar, the mixture was transferred into a small Erlenmeyer flask fitted with a condenser. The mixture, kept in an oil bath at 155° for an hour, appeared as a dark syrup, which was dissolved in a small quantity of absolute ethanol and filtered from the sodium chloride formed during the esterification. The alcoholic solution was poured into a large excess of ether, whereupon a yellow syrup separated. After standing overnight, the mother liquor was discarded and the syrup was dissolved in about 6 ml. acetone and poured into 100 ml. of light petroleum. A whitish emulsion was obtained, from which, after several hours, drops of a yellowish syrup separated. After standing overnight, the mother liquor was discarded and the semi-crystalline layer was redissolved in acetone and again precipitated by the addition of light petroleum. After this procedure had been repeated several times all the material was obtained crystalline. It was then washed with light petroleum and dried in a vacuum desiccator over sulphuric acid and paraffin. Yield: 6 g. For all analyses and enzyme tests this product was recrystallized twice from absolute ethanol by addition of ether. (Recrystallization from acetone by precipitation with light petroleum gave material yielding unsatisfactory elementary analyses.) It should be noted that once the product has been obtained crystalline, it may be recrystallized very easily. It is insoluble in ethyl acetate, ether, light petroleum or cold water and dissolves easily in hot acetone or ethanol. For analysis it was dried to constant weight over P_2O_5 at the b.p. of chloroform. The dry crystalline substance, m.p. 130° (uncorr.), was free of chloride and other ash. (Found: C, 57·1; H, 5·9; N, 5·4%. $C_{12}H_{15}O_5N$ requires C, 57·1; H, 5·9; N, 5·5%.)

Saponification value. The product (31·5 mg.) was dissolved in a small amount of hot water, a few drops of phenolphtalein were added and sufficient 0·02 N-NaOH to produce a weak pink colour (0·1–0·15 ml.). The solution was then heated to boiling and 0·02 N-NaOH was added drop by drop from a microburette until the pink colour remained permanently. Excess of NaOH was avoided in order to prevent the hydrolysis of the benzoyl-glycine linkage. (It was, however, established that this did not result even from addition of several ml. 0·02 N-NaOH and prolonged boiling.) Saponification value: 31·5 mg. required 6·2 ml. 0·02 N-NaOH. Calc. 6·3 ml.

Enzyme tests

Owing to the considerable lability of the ester in alkaline or acid medium, the tests could be conducted only with pancreatic lipase,* which is fairly active at pH 7. Phosphate buffer of pH 7 was employed.

Another difficulty was encountered in that the ester of glycerol with hippuric acid is insoluble in cold water or phosphate buffer. Its solution in a small quantity of ethanol, poured into phosphate buffer—enzyme mixture, formed a colloidal suspension, stabilized probably by the protein of the pancreatic extract. The same amount of ethanol was added to the controls.

Table 1. *Cleavage of hippurylglyceride by a glycerol extract of pancreatin activated by sodium taurocholate*

Solutions.

(1) 0·1023 g. hippurylglyceride dissolved in 1·1 ml. ethanol, 12 ml. phosphate buffer (Sorensen, pH 7), 0·9 ml. 1·5% sodium taurocholate, 3 ml. 33% glycerol extract of pancreatin, water to 22 ml.

(2) The same as (1) but 0·9 ml. water instead of 0·9 ml. sodium taurocholate.

(3) The same as (1) but with 3 ml. water instead of 3 ml. pancreatin extract.

(4) 1·1 ml. ethanol, 12 ml. phosphate buffer, 0·9 ml. sodium taurocholate solution, 3 ml. pancreatin extract, water to 22 ml.

(5) As (4), with 0·9 ml. water instead of 0·9 ml. sodium taurocholate.

Owing to the presence of phosphate ions, Ca-salts could not be used as activators. The use of veronal buffer, on the other hand, was excluded by its strongly inhibitory action on pancreatic lipase (Oppenheimer, 1936).

Result.

5 ml. of each mixture were titrated against 0·02 N-NaOH by Sorensen's formol-titration method.

Incubation time (min.)	Titration increase (ml. 0·02 N-NaOH)				
	(1)	(2)	(3)	(4)	(5)
90	1·40	1·20	—	0·75	0·55
180	1·90	1·50	—	—	0·25
270	2·30	2·05	—	—	—
Cleavage, corrected for controls	1·50	1·25			
	1·50	1·25			

Cleavage in (1) corresponds to 32·6%; in (2) to 27·2% of the weight amount.

No cleavage with liver esterase could be effected. For these experiments pig liver was ground in a mincing machine and mixed well with fuller's earth. Treatment in a Buchner press gave an extract which was diluted with an equal volume of water. The

* Prepared by extraction of pancreatin (Parke Davis and Co.) with 95% glycerol. It was previously established that sodium hippurate is not debenzoylated by this extract.

activity of the extract was such that 75 % of added ethyl butyrate was hydrolyzed in 3·5 hr. incubation time.

SUMMARY

1. The synthesis of an ester of glycerol with hippuric acid from glycerol chlorohydrin and sodium hippurate is described.

2. Cleavage effected by the action of a glycerol extract of pancreatin activated by sodium taurocholate amounted to 32·6 % after 270 min. The cleavage by the same extract in the absence of sodium taurocholate was 27·2 %.

3. Cleavage of hippurylglyceride was not effected by liver esterase.

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Yeast Lactic Dehydrogenase and Cytochrome b_2

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The dehydrogenases fall into three distinct groups, according to the nature of the intermediate hydrogen acceptors through which they react with O_2 (cf. Green & Brosteaux, 1936):

Group I, dehydrogenases which depend on the presence of one or other of the nicotinic amide nucleotides (coenzymes I and II). Through the latter substances, which act as intermediate acceptors or hydrogen carriers, they react with certain flavoproteins and so with cytochrome *c* and O_2. They cannot, however, react directly with flavoproteins, cytochrome or O_2, or with artificial acceptors like methylene blue.

Group II, flavoproteins, which do not require the addition of coenzymes, the prosthetic flavin group itself acting as intermediate acceptor. In some cases (aerobic dehydrogenases) this group can react directly with O_2; in other cases (diaphorases) the mediation of the cytochrome system is necessary.

Group III, the 'cytochrome-reducing dehydrogenases', so-called because (at any rate in the unpurified state) they readily reduce cytochrome *c*, though this may not be a direct reaction. These enzymes have a number of properties in common which distinguish them from those of the other groups. They do not depend on soluble coenzymes and readily reduce methylene blue in their absence.

Several dehydrogenases of group I have been isolated in the pure state as crystalline proteins. They do not contain any coloured prosthetic group. Several enzymes of group II have been obtained in a state approximating to purity. None of the enzymes of group III, on the other hand, has previously been isolated or purified to any great extent, and consequently the nature of the active groups of these enzymes is unknown. The reason for this is that almost all the enzymes of this class are attached to the insoluble part of the cell-structure and until they can be detached a high degree of purification is hardly possible.

Among the chief enzymes of group III are the succinic dehydrogenase, the lactic dehydrogenase of yeast (that of animal tissues is a quite distinct enzyme belonging to group I), the insoluble glycerophosphate dehydrogenase of animal tissues (the soluble glycerophosphate enzyme is again a different enzyme of group I) and probably choline dehydrogenase. The yeast lactic enzyme is of special interest because it is freely soluble and therefore its purification and isolation may be attempted with some hope of success. We undertook this with the object of obtaining information on the chemical nature of the enzyme and especially of its prosthetic group, if any, and also of studying the manner in which the purified enzyme reacts with cytochrome and other hydrogen acceptors.

Lactic dehydrogenase was first extracted from yeast by Bernheim (1928), who treated Delft baker's yeast with acetone and extracted the dry residue with alkaline phosphate solutions. Several later workers have failed to obtain active solutions in this way; the reason for this we have found to be that the acetone yeast is usually acid enough to neutralize the alkaline phosphate. If the solution is kept alkaline, Bernheim's results can be repeated.

* Preliminary notes on certain parts of this work have been published in *Nature* by Dixon & Zerfas (1939), Bach, Dixon & Keilin (1942) and Bach, Dixon & Zerfas (1942).

Even under the best conditions, however, only a small part of the enzyme is extracted by this method, and the activity of the solutions is not great. Much better results are given by autolysis under certain conditions, and after purification and concentration we have obtained solutions some thousands of times as active as those described previously. The final degree of purification, calculated on a dry weight basis with respect to the original Lebedew juice, was some 100 times; the purification in relation to the original yeast cannot be definitely stated, as it is difficult to determine the proportion of the total enzyme extracted, but it was probably over 1000-fold.

At this stage the enzyme was still not pure, but unfortunately the work had to be terminated, owing to war conditions, and the impossibility of obtaining further supplies of Delft yeast. Various English yeasts were tried, but they behaved differently, and although the enzyme could be prepared from some of them, the method had to be altered considerably and the difficulties then became so great that work on a sufficiently large scale was impracticable. Moreover, a higher degree of purification than this will probably be very difficult to achieve, on account of the inherent instability of the enzyme which increases in direct proportion with the purity of the preparation. The purest fractions lost their activity completely on standing for 20 min. at room temperature in neutral solution, although the Lebedew juice could be kept for many days without loss of activity. This makes further stages of purification extremely difficult. The work is therefore being reported in its present stage.

MEASUREMENT OF ENZYME ACTIVITY

The pH curve of the enzyme shows an optimum at about pH 5·2 (Fig. 1), and as instability of the enzyme seems to be least in this region, this pH was chosen for the standard activity tests. The curve is not symmetrical, owing to destruction of the enzyme below pH 4·5.

The Thunberg technique as modified by Green & Dixon (1934) offers a rapid and accurate quantitative test of the activity of the enzyme. No coenzyme or carrier is needed and the test is very simple. A stock test solution is made up by mixing 50 ml. M/2000 methylene blue, 50 ml. 10% sodium lactate and 200 ml. acetate buffer, pH 5·2. Into each Thunberg tube 3 ml. of this solution is pipetted and the enzyme solution (usually 0·1 or 0·5 ml.) is placed in the hollow stopper. After the tube is evacuated, filled with O_2-free N_2 and re-evacuated, it is placed in the water-bath at 37°, and the contents mixed after allowing about 2 min. to reach the temperature of the bath. The reduction time is measured with a stop-watch from the instant of mixing. Duplicate results agreed to within 3%, and the reciprocal of the reduction time was found to be exactly proportional to the amount of enzyme present within wide limits. 0·5 ml. of our most active solutions gave a reduction time of about 1 sec., but for best results the amount of enzyme taken should be adjusted to give a reduction time between 10 sec. and 1 min. Except in the initial stages of purification, no reduction occurred in the absence of lactate. With the yeast enzyme, unlike the muscle enzyme, it is not necessary to add any fixative for the pyruvic acid formed (e.g. HCN), as the reaction velocity is unaffected thereby.

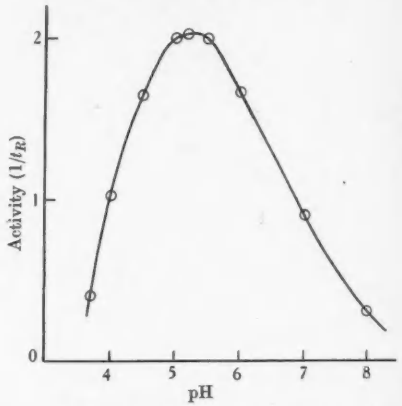


Fig. 1. pH-activity curve of enzyme.

Measurements may also be made manometrically by O_2 uptake, using methylene blue as carrier. Identical rates are obtained, as is to be expected, but the method is much less convenient.

Activity units. The reciprocal of the reduction time is a measure of the activity of a solution, i.e. of the enzyme concentration. In this paper t_R denotes the reduction time in sec. corresponding to 1 ml. of the enzyme solution. The purity is measured by the Q_{MB}, which denotes the amount of methylene blue (in μl. H_2) reduced per hr. and per mg. of protein, 1 mol. of the dye being taken as equivalent to 1 mol. of H_2.* Since the amount of methylene blue taken is equivalent to 5·6 μl. H_2, $Q_{MB} = \dfrac{60 \times 5{\cdot}6}{t_R \times w}$, where t_R is expressed in min. and w mg. of protein are taken. The protein was determined by precipitating with trichloroacetic acid, washing free from ammonium salts with dilute trichloroacetic acid and estimating N by Kjeldahl.

* In expressing methylene blue in μl. one may consider either the amount of H_2 required to reduce it or the amount of O_2 required to reoxidize it. As 1 mol. of the dye is equivalent to 1 mol. of H_2 or $\frac{1}{2}$ mol. of O_2 the results will differ by a factor of 2. The term Q_{MB} was first introduced by Dixon & Keilin (1936), who defined it with respect to O_2. It was later used by Corran, Dewan, Gordon & Green (1939*a*) as defined on the H_2 basis. This produced some confusion, especially as both papers referred to the same enzyme. In the present paper we shall use Q_{MB} as defined by Corran *et al.*, as we are dealing with an anaerobic dehydrogenase.

We define the *unit of enzyme* as the amount present in 1 mg. of a preparation with a Q value of 1. We suggest this as a rational and consistent unit which might with advantage be used for many other enzymes also. If generally adopted it would avoid the present multiplicity of arbitrary enzyme units bearing no relation to one another.

PURIFICATION OF THE ENZYME

Much preliminary work was done to determine the most promising procedures for purification, and a number of special properties of the enzyme were discovered of which good use could be made.

We were not successful in showing the presence of the enzyme in several brewer's yeasts which were tested, but large amounts could be extracted by autolysis of dried Delft baker's yeast. The enzyme began to pass out of the cells at a very early stage of the autolysis and the Q_{MB} of the extracted material rose rapidly to a maximum (see Fig. 2), after which it fell, no doubt owing to the extraction of other proteins. At the time of maximum Q_{MB} the rate of extraction of enzyme also began to decrease; thus by choosing the time suitably a starting material of high specific activity and a good yield of enzyme could both be obtained. The resulting Lebedew juice could be kept for a few days in the cold without loss of activity. Much cytochrome *c* is also extracted, but this is strongly adsorbed by alumina, which does not adsorb the enzyme from the Lebedew juice.

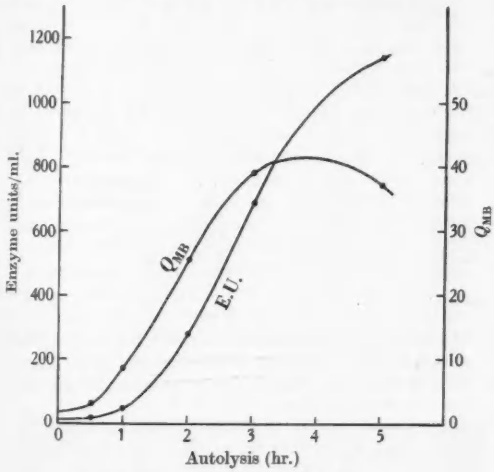


Fig. 2. Course of extraction of enzyme during autolysis of Delft yeast.

The enzyme is comparatively easily destroyed by heating, but it was found that in the presence of its substrate it could be heated to a very much higher temperature without loss of activity. This made it possible to coagulate and remove a large amount of other protein while retaining practically the whole of the enzyme. The effect of lactate was no doubt specific to this enzyme, but analogous effects probably occur with other enzymes and could probably be made use of with advantage in their preparation.

The enzyme is well adsorbed by calcium phosphate gel at pH 5–6, but no trace can be eluted with slightly alkaline phosphate solutions. This led Bernheim to believe that the enzyme was completely destroyed by the adsorption, but we have found that this is not the case. By repeatedly eluting the gel with phosphate until no further protein is extracted, large quantities of material are removed, but the enzyme remains firmly fixed to the adsorbent. It can, however, be removed by eluting with phosphate solution containing ammonium sulphate. This procedure is particularly useful in the purification.

The solution, concentrated and examined spectroscopically, showed the cytochrome *c* absorption band, but on adding lactate a new band at 5565 A. appeared. Thereafter the strength of this band in the presence of lactate ran parallel with the enzyme activity, and by spectroscopic observations the subsequent fractionations with ammonium sulphate could be carried out rapidly. The cytochrome *c* could be removed by ammonium sulphate fractionations in acid solution.

The method of purification finally worked out was based upon these properties. A number of other procedures, such as fractionation of the Lebedew juice with acetone, with lead acetate, or with ammonium sulphate seemed promising at first, but were later abandoned because they gave very unstable enzyme solutions.

Method of purification from Delft yeast. The fresh yeast cake was first uniformly broken up by pressing through an ordinary potato-masher, and was then air-dried at room temperature in batches of 1 cwt.* For each autolysis 3 kg. of dried yeast (which retained its activity for many weeks) were dispersed in 6 l. warm tap water and kept at 37° for 4½ hr. (cf. Fig. 2). 4·5 l. of cold tap water were then added, and the mixture stirred and allowed to stand 15 min. at room temperature, after which it was centrifuged for 20 min. at 3000 r.p.m. The supernatant Lebedew juice was sucked off (6250 ml., t_R 14 sec., Q_{MB} 40). The Q_{MB} at this stage varied from

* We wish to express our thanks to the Marmite Food Extract Co. Ltd. and especially to their Chief Chemist, Mr A. E. Andrews, for testing a number of different methods of drying to determine the most suitable conditions, and for carrying out the drying of the main bulk of the yeast for us.

30 to 60 in different batches. The opaque juice was coloured reddish brown, owing to the presence of cytochrome *c*, flavoproteins and yellow pigments other than flavin compounds.

To 6250 ml. Lebedew juice about 1 l. of alumina C_γ suspension (15 mg. dry weight/ml.) was added in portions, until about 15% of the enzyme had been adsorbed, the pH being kept at 5–6. The alumina, which was centrifuged off and discarded, was coloured deep red by transmitted light; the solution (7 l., t_R 17 sec.) was pure yellow and showed no absorption band of cytochrome *c*.

To 7 l. of solution, 140 ml. 50% sodium lactate were added, and the mixture was heated rapidly (in 500 ml. portions) to 53° and held at this temperature for 7 min. A bulky precipitate of coagulated protein was centrifuged off. (Without lactate the enzyme was destroyed by heating to 40°, and the amount of protein coagulated at this temperature was not large.)

(t_R 1·25 sec.). Some loss of activity occurred during this process, which required about 5 hr. Instability of the enzyme began to be noticeable at this point and thereafter increased roughly in proportion with the Q_{MB}.

The solution, which was now coloured pink, mainly owing to the concentration of small amounts of cytochrome *c* which had escaped the initial alumina adsorption, was next fractionated with ammonium sulphate as follows. The solution (A) was placed in a centrifuge tube, 25·24 g. solid ammonium sulphate (A.R.) stirred in, the precipitate (B) centrifuged down, the supernatant fluid poured off into another centrifuge tube, 5·8 g. ammonium sulphate added, and so on, as shown in Table 1. The various precipitates B–G were dissolved in water and made up to the volumes shown in col. 3. The final supernatant fluid, which was discarded, still contained much protein. Fraction E was the purest and also gave the most active solution (t_R 0·6 sec.).

Table 1. 130 *ml. of solution A, fractionation at* pH 7·2

Amm. sulph. added (g.)	Fraction	Ppt. made up to (ml.)	t_R (sec.)	Units/ml.	Total units	Q_{MB}	
25·24	B	7·5	6·1	3,300	24,800	—	discarded
+5·8	C	5	2·7	7,500	37,500	—	discarded
+5·2	D	20	1·5	13,500	269,000	1730	
+6·3	E	15	0·6	33,500	504,000	1750	mixed (=H)
+7·0	F	22	0·9	22,500	493,000	1330	
+9·6	G	3·5	3·0	6,700	23,500	—	discarded

Supernatant solution inactive.

To the resulting solution (7 l., t_R 19 sec.) was added 3·5 l. Ca phosphate gel suspension (40 mg. dry wt./ml.). After centrifuging, the supernatant solution contained only a small amount of unadsorbed enzyme (t_R 390 sec.), but much protein. The Ca phosphate was eluted five times in succession, each time with 2·5–3 l. M/15-phosphate buffer, pH 7·2. The eluates were quite inactive, but contained much protein and practically all the yellow colour. The enzyme was then eluted from the Ca phosphate by three successive extractions with pH 7·2 phosphate buffer containing 10% ammonium sulphate, 400, 1200 and 1200 ml. respectively being added. The t_R of the three eluates were 9, 8 and 24 sec. respectively. The three eluates, which were water-clear, were mixed (2800 ml.; t_R 16 sec.) and kept cold overnight without loss of activity.

The enzyme solution was then concentrated on a battery of Bechhold ultrafilters fitted with an automatic feed and with brushes lightly sweeping the surface in order to increase the rate of filtration. '3%' Schleicher collodian ultrafilter membranes were used and 100 ml. of solution could be filtered in an hour by each ultrafilter. In this way 2800 ml. (t_R 16 sec.) were concentrated to 130 ml.

In another experiment the t_R was 0·3 sec., this being the most active solution obtained in this work.

Fractions D, E and F were mixed, giving a clear red solution (H) showing a strong cytochrome *c* spectrum (Fig. 3*a*). On the addition of a small amount of lactate, however, a new band at 5565 A. instantly appeared, the spectrum then being as

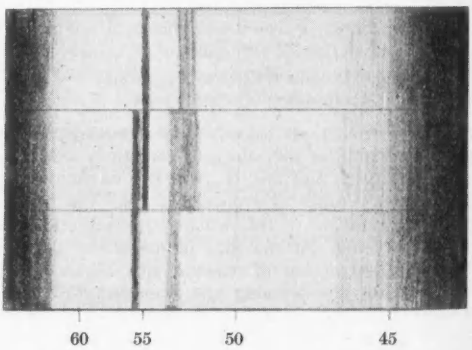


Fig. 3. Absorption spectra of purified enzyme fractions. (*a*) fraction H; (*b*) fraction H + lactate; (*c*) fraction M + lactate.

shown in Fig. 3*b*. As the band at 5565 A. in presence of lactate invariably accompanies the enzyme through all the fractionations, it can be used as a quick test for controlling the ammonium sulphate precipitations.

Preparation from Manchester yeast. When it became impossible to obtain Delft yeast, owing to war conditions, we attempted to use English baker's yeast, but found that we were unable to prepare the enzyme by the above method, on account of the much greater difficulty of extracting the enzyme from the cells. An entirely new method had to be worked out, and although this was done successfully, the new method is too laborious to be practicable for general use. As some essential data were obtained with such preparations, however, and as they yielded an important piece of evidence as to the nature of the system, the method is given here.

Table 2. *Solution H, fractionation at* pH 4·8

53 ml. solution H + 26 ml. M/5-acetate (pH 4·8) + 53 ml. water

Amm. sulph. added (g.)	Fraction	Ppt. made up to (ml.)	t_R (sec.)	Units/ml.	Total units	E_{5500}	E_{5565}	
32·0	J	8	3·4	5,900	47,500	—	0·4	discarded
+4·8	K	7	1·4	14,400	101,000	0	0·75	discarded
+3·9	L	8	0·65	31,000	248,000	0	1·32	(see Fig. 4)
+3·7	M	8	0·75	26,900	215,000	0	1·18	mixed (=Q)
+6·4	N	8·5	1·1	18,300	156,000	trace	0·97	
+7·3	P	4	8·0	2,500	10,100	0·4	0·1	discarded

Supernatant solution inactive.

The enzyme was then further purified, and the cytochrome *c* completely eliminated, by fractional precipitation by ammonium sulphate in acid solution, as follows: 53 ml. solution H (t_R 0·9 sec., Q_{MB} 1550, 1,180,000 units) was mixed with 26 ml. M/5-acetate buffer (pH 4·8) and diluted with 53 ml. water to facilitate separation of the precipitates. It was then separated into six fractions (J–P) with ammonium sulphate, as shown in Table 2. The first fraction gave a slightly opalescent, pale and not very active solution; the next fractions gave perfectly clear, deep-red and very active solutions, quite free from all traces of cytochrome *c*, but showing on reduction a very strong 5565 band, as shown in Fig. 3*c*; the last fraction was much less active, and had a weak 5565 band, but a stronger cytochrome *c* band at 5500 A.; the final supernatant fluid showed a strong spectrum of pure cytochrome *c* and was quite inactive. The extinctions ($E = \log_{10} I_0/I$) of these solutions at 5500 and 5565 A. were measured in a Hilger-Nutting spectrophotometer with a 2 cm. cell, and are shown in Table 2 (cf. also Fig. 5). The absorption spectrum curve of the most active solution (fraction L) was determined in the same instrument, and is shown in Fig. 4. Fractions L, M and N were mixed, giving solution Q (24 ml., t_R 0·8 sec., 600,000 units). The instability of the enzyme was now so marked that further attempts at purification resulted in the loss of most of the activity. The Q_{MB} of solution Q was unfortunately not exactly determined in this particular preparation, but in other similar preparations it was about 2500. The progress of the purification is shown in Table 3. The highest Q_{MB} obtained by us was nearly 3000, after adsorbing the enzyme first on calcium phosphate and then on alumina, followed by ammonium sulphate fractionation, but the yield was much smaller than by the method given above.

Table 3. *Course of purification from Delft yeast*

Stage	Volume (ml.)	t_R (sec.)	Q_{MB}	Total units
Lebedew juice	6250	14	40	9,000,000
After alumina	7000	17	—	8,300,000
After heating	7000	19	—	7,430,000
Eluate from Ca phosphate	2800	16	—	3,530,000
Ultrafilter concentrate	130	1·25	—	2,100,000
$(NH_4)_2SO_4$ fraction H	57	0·9	1550	1,280,000
Acid $(NH_4)_2SO_4$ fraction Q	24	0·8	*c.* 2500	600,000

20 kg. fresh Manchester yeast were plasmolyzed by adding solid NaCl. The liquefied mass (17 l.) was ground in a high-speed metal ball-mill in batches of 130 ml. for 20 min. each, in order to break the cells. It was then incubated at 37° for 3 hr. with mechanical stirring, during which time toluene was slowly added up to a final concentration of 7%. 9 l. of M/15-phosphate buffer (pH 7·2) were then added and the whole was centrifuged, giving 12 l. of Lebedew juice (t_R 105 sec.). The enzyme from 11 l. of juice was then adsorbed on 550 g. of kaolin, which was centrifuged off and eluted with 1·8 l. M/15-phosphate buffer (pH 7·2) containing 20% of ammonium sulphate (t_R of eluate 45 sec.). On standing, some activity was lost. 600 g. solid ammonium sulphate were added, the precipitate filtered off after standing overnight in the refrigerator, dissolved in water and dialyzed for 3 hr. (1100 ml., t_R 35 sec.). 165 ml. calcium phosphate gel were then added to adsorb the enzyme washed with 600 ml. phosphate buffer (pH 7·2) and eluted with 700 ml. of the same buffer containing 20% of ammonium sulphate

(t_R 28 sec.). On standing, half the activity was lost (t_R 55 sec.). The solution was saturated with ammonium sulphate and placed in the refrigerator. The precipitate which formed overnight was taken up in 10 ml. of water (t_R 1·8 sec.). On adding lactate to this solution the absorption band at 5565 A. was clearly seen as in the enzyme preparation from Delft yeast. From this point the purification followed the procedure of the method already described, by fractional precipitation with ammonium sulphate, first at pH 7·2, then at pH 4·8. The final fraction (T), corresponding to fraction L of the previous preparation, had about the same Q_{MB}, representing a purification of more than 200 times with respect to the Lebedew juice. It was used for spectrophotometric determinations needed for calculations given later. The general course of the purification by this method is shown in Table 4.

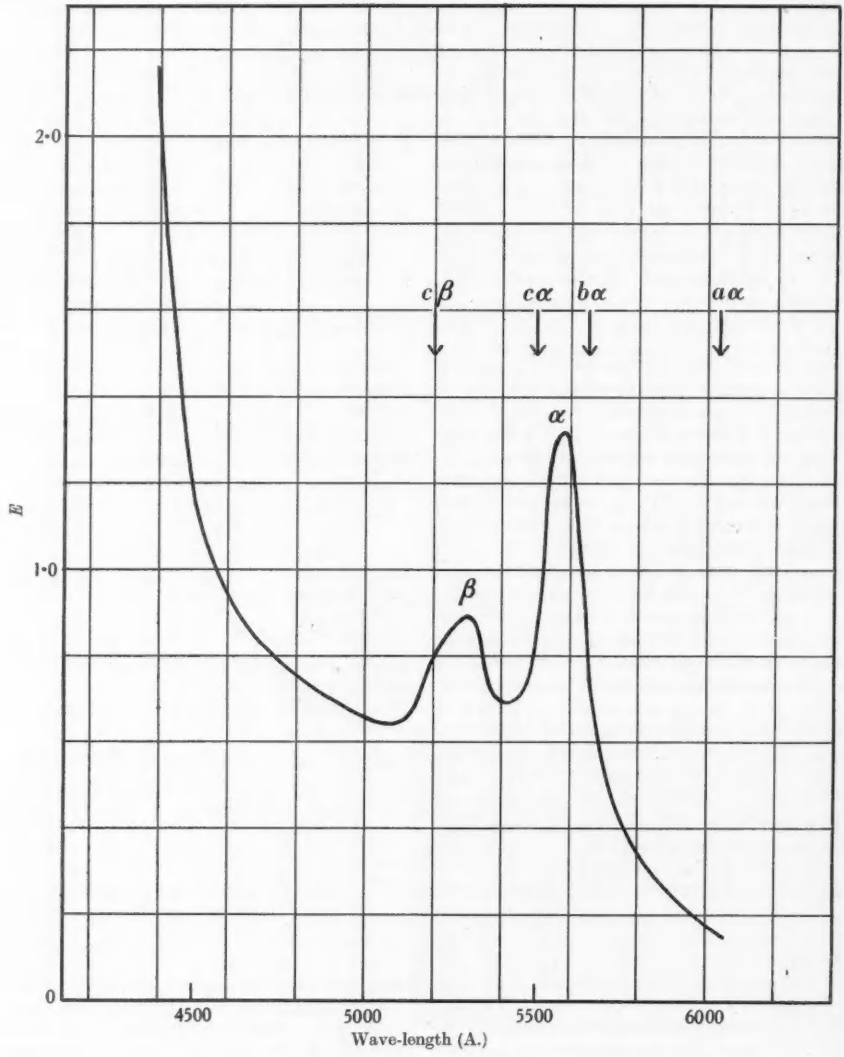


Fig. 4. Absorption spectrum curve of cytochrome b_2.

Table 4. *Course of purification from Manchester yeast*

Stage	Volume (ml.)	t_R (sec.)	Q_{MB}	Total units
Lebedew juice	12,000	105	10·5	2,300,000
Eluate from kaolin	1,800	45	55	800,000
Pptd. and dialyzed	1,100	35	250	630,000
Eluate from Ca phosphate	700	28	1100	500,000
Eluate from Ca phosphate (after standing)	700	55	556	257,000
$(NH_4)_2SO_4$ ppt.	10	1·8	—	110,000
Acid $(NH_4)_2SO_4$ fraction T	4	1·7	2280	47,500

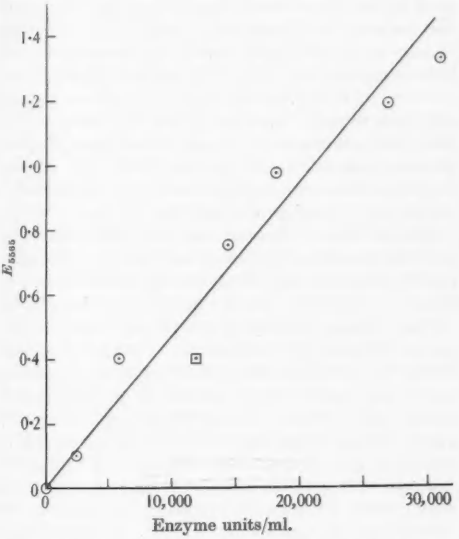


Fig. 5. Relation between enzyme activity and intensity of cytochrome b_2 absorption band in different fractions. The circles represent points given by fractions J–P from Delft yeast, the square was given by fraction T from Manchester yeast.

CYTOCHROME b_2

The coloured substance which accompanies the enzyme and is responsible for the absorption spectrum shown in Fig. 3*c* and Fig. 4 is evidently a haemochromogen protein. Further examination has shown that it is definitely a cytochrome, though not identical with any of those previously known. As it is most closely allied to cytochrome *b*, we have proposed the name cytochrome b_2, in order to distinguish it from other components and to avoid any implication as to its identity with the enzyme. (The name cytochrome b_1 has been given to a modified cytochrome present in certain bacteria; Keilin, 1934.)

The new cytochrome differs from those previously described, with the exception of cytochrome *c*, in being extremely soluble in water. The α band of its spectrum, as will be seen in Fig. 4, lies almost midway between those of cytochromes *b* and *c*. The Hartridge reversion spectroscope gives its position as 5563 A. It is not visible in the absorption spectrum of yeast as its concentration there is far too low.

The absorption spectrum, which is that of the reduced form of cytochrome b_2, appears instantly on the addition of either lactate or hyposulphite ($Na_2S_2O_4$). The reduced form is slowly autoxidizable and in either case the bands gradually disappear on shaking with air, provided that only a small amount of reducer has been added. When the enzyme activity of the solution has been lost after keeping, the bands do not appear on adding lactate, but they appear, apparently unchanged, on reduction by hyposulphite.

Prof. Keilin very kindly made an examination of fraction L and made the following observations. The preparation was free from cytochrome *c*. On denaturation with dilute NaOH and addition of pyridine and hyposulphite the haematin of cytochrome b_2 was converted into the corresponding pyridine haemochromogen. The spectrum of this was identical with that given by pyridine protohaemochromogen, showing that cytochrome b_2 has protohaematin as its prosthetic group, like cytochrome *b* and unlike cytochromes *a* and *c*. It resembles cytochrome *b* also in being slightly autoxidizable. No change in the spectrum was produced by saturating with carbon monoxide in neutral solution, showing that, like cytochromes *a*, *b* and *c* but unlike other haemochromogens, cytochrome b_2 does not combine with CO under these conditions. When the solution was frozen in liquid air the absorption bands became much sharper and the α band was then seen to be double; this has also been seen with some other haemochromogens. When a strong solution of oxidized cytochrome *c* was added to the preparation, followed by lactate, the cytochrome *c* became reduced as well as the cytochrome b_2.

By converting the cytochrome b_2 into pyridine haemochromogen, as described above, and comparing the solution with standard pyridine haemochromogen solutions by the spectroscopic method described by Keilin (1933, 1941), its concentration may be accurately determined. In this way Prof. Keilin kindly determined the concentration of cytochrome b_2 haematin in solution T, the final fraction from the Manchester yeast preparation. This was found to be equivalent to 0·0040 mg. haemin/ml. The absorption curve of this solution, after reduction with hyposulphite, had been determined in the spectrophotometer and E_{5565} estimated as 0·4, using

a 2 cm. cell. The absorption coefficient β (where $\beta=\frac{1}{cd}\ln\frac{I_0}{I}$, and c is the concentration and d the thickness of the cell) corresponding to the α band is therefore given by

$$\beta_{5565}=\frac{2{\cdot}3\times1000\times652\times0{\cdot}4}{2\times0{\cdot}0040}=0{\cdot}75\times10^8\ \frac{\text{cm.}^2}{\text{g. atom Fe}}.$$

This is of the same order as that of the α band of cytochrome *c*.

FURTHER PROPERTIES OF THE ENZYME

Instability. The greatest difficulty in this work was caused by the increasing instability of the enzyme as purification proceeds, and until some means of preventing it is found, it is unlikely that the purification will be carried much further. Many unsuccessful attempts to stabilize the enzyme were made by the recognized methods. The use of glass-distilled water throughout, alterations of pH, and the addition of glycine, gum arabic, lactose, various proteins, cyanide, cysteine or glutathione, were all ineffective. The possibility that the destruction might be due to another enzyme (e.g. a proteinase) was considered and many attempts were made to separate it from the lactic enzyme but without success. Indeed, if this were the cause, the properties of the two enzymes would have to be remarkably similar to account for the fact that the more the lactic enzyme is purified, the more rapidly is it destroyed.

Two substances had a marked stabilizing effect, namely lactate, the substrate of the enzyme, as already mentioned, and ammonium sulphate in high concentration. By adding both together, preparations of moderate purity may be made almost completely stable. Thus rather impure preparations (Q_{MB} up to 1000) are fairly stable; moderately purified fractions (Q_{MB} 1000–2000) lose most of their activity in a few hours, but can be very largely stabilized by ammonium sulphate in presence of lactate; and the purest fractions (Q_{MB} 2000–3000) become inactive in about 20 min. without ammonium sulphate, and even when kept saturated with ammonium sulphate at 0° in neutral solution lose 90 % of their activity in a few hours. This makes it extremely difficult to carry the purification further.

For the same reason it was not possible to investigate the homogeneity of the final fractions in the Tiselius apparatus, since this requires a preliminary dialysis for some hours against dilute buffer solution, during which the enzyme would be completely destroyed.

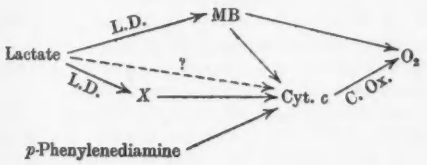
Coenzyme. It seems certain that the lactic dehydrogenase of yeast, unlike that of muscle, does not involve any dissociable coenzyme. The method of purification is such that any such coenzymes would be very completely removed, e.g. by the exhaustive washing of the enzyme adsorbed on calcium phosphate. The purified preparations are, in fact, quite free from all traces of the well-known coenzymes, and we have been unable to detect them even in small traces by delicate enzyme tests, both before and after denaturing the solutions by heat or acid. Actually they are found to be removed in the early stages of the purification. The addition of the usual coenzymes does not increase the activity of the enzyme. It is true that activity is lost by dialysis, but the non-dialyzed solutions also lose activity at the same time, so that the loss is not due to dialysis. As already mentioned, fractions of only moderate purity are completely stabilized by ammonium sulphate, and such solutions may be dialyzed for many days against ammonium sulphate solutions without loss of activity. Furthermore, we have attempted in many ways to resolve our enzyme preparations into two fractions having a greater activity when mixed than they have separately, and we have been unable to do so.

Flavoprotein. The final fractions obtained by the method of purification described above are not altogether free from flavoprotein. We think, however, that it is unlikely that flavoprotein plays any part in the system. There is no parallelism between the amount present and the activity, and by a modified method of preparation we were able to obtain highly active solutions in which no traces of flavoprotein could be detected. By repeatedly adsorbing the enzyme on alumina, followed by ammonium sulphate fractionation, active solutions could be obtained (though with a much smaller yield and containing much more cytochrome *c*) which (*a*) showed no decrease of absorption at 4500A. on addition of hyposulphite, (*b*) after denaturation gave no increase of activity with the flavin-freed *d*-amino-acid oxidase, and (*c*) showed no trace of fluorescence in ultra-violet light, either before or after denaturation by heat or acid. Test (*a*) is not so sensitive as the other two. Test (*b*), though sensitive, detects only flavoproteins containing flavin dinucleotide. Test (*c*) is highly sensitive, and although not all flavoproteins fluoresce, they all give a strong fluorescence when the prosthetic group has been split off by denaturation. It is clear that if the methylene blue reduction by the system takes place through the mediation of a flavoprotein, it must have a catalytic activity far higher than that of any of the flavoproteins known at present. Whether the reduction of cytochrome *c* by the system involves a flavoprotein cannot as yet be definitely stated.

Reaction with cytochrome c. In their preliminary note, Dixon & Zerfas (1939) stated that the yeast lactic enzyme loses its power of reducing cytochrome *c* when it is purified, whereas it was stated above

that the purest fractions could be seen spectroscopically to reduce cytochrome *c* rapidly. The explanation of this apparent discrepancy is that although the enzyme can apparently react with cytochrome *c* to some extent, the rate of this reaction was too small to be effective in catalyzing any appreciable O_2 uptake when the partially purified enzyme solutions of Dixon & Zerfas were used. When, however, the enzyme is highly concentrated, as in the extremely active solutions now obtained (which are far more concentrated than any available in 1939), the reaction rate is sufficient to show a rapid reduction of cytochrome *c* under the spectroscope. We cannot say at present whether the completely pure enzyme would be able to react with cytochrome *c*, or whether the reaction is due to some additional factor still present in the final solutions.

Evidence confirming the view of Dixon & Zerfas, that the rapid reduction of cytochrome *c* by crude preparations of the enzyme depends on the presence of an additional factor which is removed in the purification and which is not required for the reduction of methylene blue, has been obtained, using a purified preparation from Manchester yeast. This was prepared as described above, but was not taken to the final stages of purification. The rates of reduction of cytochrome *c* and methylene blue by the system were conveniently compared by O_2 uptake measurements after the addition of either cytochrome *c* plus excess of cytochrome oxidase or of methylene blue, since the reduced cytochrome and the reduced methylene blue are both reoxidized with uptake of O_2. The reactions taking place are shown in the diagram, the arrows showing the direction of hydrogen transfer.



On carrying out the experiment at pH 5·2, the optimum pH of the lactic dehydrogenase, no O_2 uptake was obtained in either case. With methylene blue, this was due to the failure of the reduced dye to react with O_2 at the acid pH, as shown by the fact that the solution was completely decolorized. The addition of cytochrome oxidase, however, catalyzed the reoxidation of the dye and a rapid O_2 uptake was then obtained. The absence of O_2 uptake with the cytochrome system without methylene blue, however, did not necessarily show that the cytochrome was not reduced by the lactic dehydrogenase system, as the cytochrome-oxidase system was found to be practically inactive at this pH, giving no O_2 uptake with *p*-phenylenediamine. It is not clear why the oxidase should be able to oxidize leucomethylene blue but not *p*-phenylenediamine at this pH.

At pH 7·6 these difficulties were avoided, and the lactic dehydrogenase still retained sufficient activity at this pH for the O_2 uptake measurements, while the cytochrome oxidase gave a rapid O_2 uptake with *p*-phenylenediamine. Fig. 6 shows results obtained under these conditions, and it will be seen

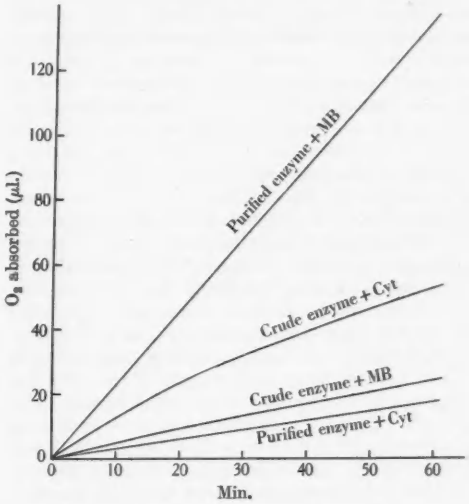


Fig. 6. Reaction of crude and purified enzyme with cytochrome *c* and with methylene blue.

that with the purified enzyme the reaction with cytochrome is extremely slow compared with the reaction with methylene blue. Similar results have been obtained with other purified preparations. With cruder preparations, on the other hand, it will be seen that the rates are reversed and the reaction with cytochrome is much more rapid than the reaction with methylene blue. This is evidence for the presence in the unpurified preparations of a factor (*X*) catalyzing the reaction of the lactic system with cytochrome, but not required for the reaction with methylene blue. The experiments of Fig. 6 were done at the same time and with the same cytochrome oxidase preparation.

DISCUSSION

This work was undertaken mainly with the object of throwing some light on the chemical nature of dehydrogenases of this group, which are not flavoproteins and do not depend on coenzyme I or II.

The most interesting question which arises is, therefore, whether the yeast lactic dehydrogenase is identical with the cytochrome b_2 which invariably accompanies it or not. If it were identical, this would be a new kind of function for a haematin-protein, but there is no inherent improbability in such an assumption and cases of reactions between methylene blue and haemochromogens are already known.

Evidence for identity is provided by the close similarity in the properties of the enzyme and cytochrome b_2, which causes the pigment to accompany the enzyme activity through all the fractionations involved in the purification, so that the absorption band can be used as a quick test for locating the enzyme during fractionation. Moreover, there is a quantitative parallelism between the strength of the absorption band and the enzyme activity in different solutions, as shown in Fig. 5. We have noticed in many preparations that, as the enzyme is concentrated, the first appearance of the band occurs as the t_R approaches 5 sec. The preparation from Manchester yeast yielded strong confirmatory evidence, for in spite of the fact that the method of preparation was almost entirely changed from that previously used, the same band appeared when about the same activity was reached. Moreover, the strength of the band in the final fraction T was approximately that predicted from the activity: E_{5565} was found to be 0·4 for this solution, using a 2 cm. cell, and this gives the point shown by a square in Fig. 5. It will be seen that this falls fairly close to the line previously obtained. If cytochrome b_2 has no connexion with the lactic dehydrogenase system this would indeed be a most remarkable coincidence.

It is true that the same absorption band can be seen when the solution is reduced with hyposulphite after the enzyme has become inactivated on standing, but this is not necessarily an argument against identity, since the lactate-activating group in the enzyme might become inactivated without affecting the haematin group. A somewhat similar case is that of xanthine oxidase, in which, according to Corran *et al.* (1939*a*), the xanthine-activating part of the enzyme may become inactivated while the flavin group remains intact.

It seems clear, therefore, that cytochrome b_2 forms an essential part of the catalyst causing the reduction of methylene blue by lactate. In this connexion it is very interesting to note that in heart muscle cytochrome *b* is intimately associated with the succinic dehydrogenase, a dehydrogenase of the same class as the yeast lactic enzyme (Keilin & Hartree, 1939). It is not impossible, therefore, that the enzymes of this group are cytochromes of the *b* type.

The available evidence, however, is insufficient to show whether cytochrome b_2 is identical with the lactic dehydrogenase itself, or whether it forms an intermediate carrier acting between the dehydrogenase and methylene blue. The proportionality between the rate of reduction of the dye and the amount of cytochrome b_2 present could be accounted for on either view. If the pigment were identical with the enzyme a proportionality would obviously be expected; if it were a carrier this would also be the case, provided that the dehydrogenase itself is always present in excess, so that the carrier is the limiting factor, and provided that the methylene blue reacts only through the carrier. It is not easy to see, however, why the dehydrogenase should still be present in excess after the whole fractionation procedure, resulting in a high degree of purification and a great increase in activity.

An argument for the carrier hypothesis might at first sight seem to be provided by an observation by Haas, Horecker & Hogness. They observed (1940) 'a small band at 557 mμ' in impure preparations of their cytochrome *c* reductase from yeast. Later (1942), after the appearance of our preliminary note on this work, they assumed that the substance responsible for this absorption band was identical with cytochrome b_2.* They found that the substance was reduced on adding hexosemonophosphate, hexosemonophosphate dehydrogenase and coenzyme II to the reductase preparation. If we assume that this observation refers to cytochrome b_2, it would seem to suggest that it can act as a carrier in the hexosemonophosphate system as well as in the lactate system. In reality, however, the two reactions are quite different. In the former case the reaction consists in the oxidation of reduced coenzyme II, while in the latter case this coenzyme is not a component of the system and the reaction consists in the linking of lactate with methylene blue. It does not seem impossible that the impure reductase preparation may have contained lactic dehydrogenase and also enzymes capable of forming traces of lactate from the hexosephosphate, in which case cytochrome b_2 would certainly be reduced. The traces of lactate required to produce the 5565 band in our preparations are extremely small, and a momentary contact with the human skin is quite sufficient to cause its almost instantaneous appearance.

* It is doubtful whether the evidence is adequate for such an identification. Their published spectrophotometric curve (1942) shows the band only as a small rise on a curve indicating a considerable general absorption. Its intensity is less than one-fiftieth of the α band shown in Fig. 4, and there is no trace whatever of any β band. Under these circumstances it is not easy to determine the position of the band with precision, but even if it were coincident with the α band of cytochrome b_2 it would not necessarily prove identity. For example, the α bands of cytochromes *a* and a_3 coincide, although they are not identical. It is not stated whether the preparation contained lactic dehydrogenase.

It is clear that it must still remain an open question whether cytochrome b_2 is identical with the yeast lactic dehydrogenase or whether it forms an essential intermediate carrier in the system.

In either case it is possible to calculate the catalytic activity of pure cytochrome b_2 and to draw certain conclusions. If we consider fraction T, which contained 0·0040 mg. cytochrome b_2 haemin/ml., as already stated, we can calculate the Turnover Number (T.N., i.e. the number of times it must be reduced and oxidized per minute in order to account for its observed catalytic activity) as follows. Bearing in mind that 1 mol. of haemin requires 1 equivalent of H for reduction, while 1 mol. of methylene blue requires 2 equivalents, we see that 1 ml. of M/1000-methylene blue is equivalent to 1·30 mg. of haemin (M.W. 652). Thus 0·5 ml. M/2000 methylene blue (the amount taken in the t_R test) is equivalent to 0·325 mg. haemin. This was reduced by 1 ml. of solution T in 1·7 sec. or 0·028 min., so that the amount per min. would be equivalent to 11·6 mg. haemin. The turnover number is then given by $\text{T.N.} = \frac{11{\cdot}6}{0{\cdot}0040} = 2900$. This is an entirely reasonable value, and may be compared with the T.N. of pure cytochrome *c*, which is 1400 in the succinic system *in vitro* and 3800 in the living yeast cell (Keilin & Hartree, 1940). The T.N. of diaphorase varies from 1000 to 8000, according to the dilution (Corran, Green & Straub, 1939*b*).

As 1 ml. of solution T contained 11,900 enzyme units, 1 mg. of haemin would correspond to 3,000,000 units within the limit of error.

The Q_{MB} of the pure catalyst depends on the molecular weight assumed. If the M.W. per haematin group is 68,000, as in catalase, the Q_{MB} when pure will be $\frac{3{,}000{,}000 \times 652}{68{,}000} = 29{,}000$. The purity of our best fractions ($Q_{MB} = 3000$) would then be a little over 10 %. If, on the other hand, the M.W. per haemin group is 17,000, as in cytochrome *c* and haemoglobin, the Q_{MB} when pure would be $\frac{3{,}000{,}000 \times 652}{17{,}000} = 115{,}000$ and the purity of our best fractions would be only 2·5 %. For comparison with these figures we may mention that pure crystalline lactic dehydrogenase from heart muscle, a dehydrogenase of fairly average activity, appears from the results of Straub (1940) to have a Q_{MB} of 60,000, assuming that the O_2 taken up was converted into H_2O_2, as it probably would be under Straub's conditions. Other dehydrogenases are known with up to twice this activity, as well as some with lower activities. Thus calculations based on the assumption that cytochrome b_2 is responsible for the catalytic activity of the yeast lactic dehydrogenase lead to quite reasonable figures.

It can be calculated from these results that the Lebedew juice obtained from 1 kg. yeast (fresh weight) contains on the average only 5–10 mg. of the pure protein.

SUMMARY

1. The lactic dehydrogenase of yeast (which belongs to the 'cytochrome-reducing' group) has been extracted and purified; solutions some thousands of times as active as those previously known have been obtained. The enzyme is still far from pure, but increasing instability makes further purification difficult.

2. The enzyme does not depend on soluble coenzymes, nor apparently on flavin groups, for its activity.

3. A new cytochrome ('cytochrome b_2'), freely soluble in water, has been found in the concentrated enzyme fractions. The nature of its prosthetic group, its absorption spectrum and other properties have been determined.

4. Evidence is presented showing that cytochrome b_2 forms an essential part of the enzyme system, either as the dehydrogenase itself or as an essential intermediate carrier between lactate and methylene blue.

5. There is evidence that an additional factor is necessary to enable the system to react with cytochrome *c*.

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Inhibition of Urease by Copper Salts in the Presence of Ascorbic Acid and Related Substances

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Elson (1943) found that urease is inhibited by small amounts of ascorbic acid and that this inhibition is eliminated in the presence of cysteine. Quastel (1943) suggested that the inhibition was due to a reaction between dehydroascorbic acid and an —SH group of the enzyme, and that the protective effect of thiol compounds was due to the fact that they reduced dehydroascorbic acid. Giri & Seshagiri Rao (1944), however, did not find that dehydroascorbic acid inhibited urease, and since they observed that there was no inhibition of the enzyme by ascorbic acid in the presence of substances which form complexes with copper (8-hydroxyquinoline and diethyl-dithio-carbamate), they suggested that the inhibition might be due to the formation of intermediary substances like Cu_2O, produced during the oxidation of the vitamin. Our experiments show that neither ascorbic, nor its oxidation products, dehydroascorbic acid or 2:3-diketo-gulonic acid, themselves inhibit urease. Ascorbic acid and 2:3-diketo-gulonic acid, however (but not dehydroascorbic acid), act as inhibitors in the presence of Cu^{++}. Cu^{+} inhibits urease to a much greater extent than does Cu^{++}, and the increased inhibition observed when ascorbic acid is added to a solution of the enzyme containing Cu^{++} is due, it is therefore concluded, to the reduction of Cu^{++} to Cu^{+}.

METHODS

Reagents. Salts were A.R. and glass-distilled water was used for all solutions. Copper was added either as chloride or sulphate.

Urease. Two types of enzyme preparation were used. The first was crystalline urease prepared according to the method of Sumner (1926). The second was made by extracting 1 g. of Arlco jack bean meal with 40 ml. of glass-distilled water; insoluble material was separated by centrifuging and the centrifugate dialyzed for 24 hr. against glass-distilled water at 0°. The former preparation is referred to in the text as crystalline urease and the latter as dialyzed enzyme. No major differences were observed in the behaviour of these two enzyme solutions.

Urease activity. The activity of the enzyme was tested by its reaction with urea in phosphate buffer (pH 6·8), containing 5·4% (w/v) Na_2HPO_4 and 4·2% (w/v) KH_2PO_4. Each 10 ml. of reaction mixture contained 5 ml. of buffer solution, the appropriate amount of enzyme, and 1% (w/v) of urea. Samples for analysis were removed at intervals over a period of 20 min., the reaction was arrested by the addition of HCl, and the ammonia formed estimated either by Van Slyke & Cullen's method of aeration or by nesslerization. The concentration of enzyme used was adjusted so that within a period of 20 min. about 1–1·4 mg. of NH_3/ml. of reaction mixture was formed.

Ascorbic acid and related substances were added to a solution of total volume 9 ml., containing 5 ml. of the phosphate buffer and 1 ml. of a suitable concentration of urease solution. The enzymic activity was then tested as described, at any given time, after the addition of 1 ml. of a 10% urea solution. All experiments were carried out at 20°. The values in the tables under the heading 'inhibition of urease activity' refer, unless otherwise stated, to the suppression of NH_3 formation over a period of 20 min.

Dehydroascorbic acid was prepared by shaking ascorbic acid, dissolved in 0·05 M-phosphate-citric acid buffer (McIlvaine), pH 3·5, with activated charcoal. Before use the charcoal was extracted with boiling N-HCl, re-extracted with distilled water and washed with water to remove metallic impurities. Five mg. of ascorbic acid, dissolved in 50 ml. of the buffer solution, were shaken for 3–5 min. with 0·4 g. of the charcoal and the latter removed by filtration. The concentration of dehydroascorbic acid so formed was determined by reduction with H_2S and titration against indophenol in the usual way. For studying the effect of this dehydroascorbic acid solution on the enzyme the concentration of the phosphate-citric acid buffer solution added to the control was equal to that used in the preparation of the dehydroascorbic acid solution.

2:3-*Diketo-gulonic acid* was used in the form of the mono-sodium salt, kindly given by Messrs Roche Products, Ltd., and as the calcium salt, isolated according to the method described by Penney & Zilva (1943).

RESULTS

Exp. 1. *Influence of copper.* Ascorbic acid had no inhibitory influence on urease in the absence of ionized copper. The results of experiments (Table 1) with a dialyzed enzyme preparation showed no inhibitory effect of ascorbic acid in concentrations of 20–200 μg./ml. in the absence of copper, but marked inhibitory effects when it was added. An apparently anomalous finding was that when a more purified crystalline enzyme preparation was used, ascorbic acid caused some inhibition even in the absence of added copper. This inhibition was, however, due to traces of copper in the reagents used,* for the inhibition was abolished in the presence of

* Phosphate buffer-urea solution used in these experiments contained approximately 0·05–0·1 μg. of copper/ml. as estimated by sodium diethyl-dithio-carbamate reagent.

Table 1. *Influence of copper on the inhibition of urease by ascorbic acid*

Test no.	Enzyme preparation used	Final concentration of copper added (μg./ml.)	Final concentration of ascorbic acid added (μg./ml.)	Inhibition of urease activity (%)
1	Dialyzed	0	2·5	0
		0	5	0
		0	10	0
		0	20	1
		0	200	0
2	Dialyzed	0·2	0	5
		0·2	2·5	17
		0·2	5	26
		0·2	10	36
		0·2	20	42
3	Dialyzed	1	0	24
		1	10	96
		1	20	100
4	Crystalline	0*	2·5	10
		0	5	25
		0	10	32
		0	20	44
		0†	2·5	0
		0†	5	0
		0†	10	0
		0†	20	0
		0‡	5	0
		0‡	10	0
5	Crystalline	0·1	0	7
		0·1	10	86
		1	0	39
		1	10	100

* Solution contained approx. 0·05–0·1 μg./ml. of copper as impurity.

† Solutions contained 0·01 M-pyrophosphate.

‡ Solutions contained 0·01% (w/v) sodium diethyl-dithio-carbamate.

pyrophosphate, or sodium diethyl-dithio-carbamate, reagents which are known to form complexes with copper. The most probable explanation of this apparent anomaly is that, on purification of the enzyme, substances like thiol compounds and proteins, which are able to form complexes with copper, are removed. Thus with the crude enzyme any traces of copper present in the reagents are rendered inactive, but this is not so in the case of the pure enzyme, and the copper is thus able to exert its full effect.

The results also indicate that the magnitude of the inhibitory action of ascorbic acid is conditioned by, and increases with, the concentration of Cu^{++} (Table 1, tests 2, 3 and 5).

Exp. 2. *Inhibition under anaerobic and aerobic conditions.* On the basis of the suggestion advanced by Quastel the function of copper would be to catalyze the aerobic production of dehydroascorbic acid from ascorbic acid. The results of experiments (Table 2) suggest that such an explanation is unlikely. Ascorbic acid was added to urease in the presence of Cu^{++}, and the solutions left for periods varying from 0 to 90 min. before testing the activity of the enzyme. The inhibitory effect was found to be as marked under anaerobic as under aerobic conditions, despite the fact that the oxidation of ascorbic acid and formation of dehydroascorbic acid was largely prevented in the former case. However, anaerobic conditions will not prevent the reduction of Cu^{++} by ascorbic acid and the possibility existed that the small amounts of dehydroascorbic acid ($<0·025$ μg./ml.) so formed might have been sufficient to account for the inhibition.

Exp. 3. *Inhibitory effect of substances formed from dehydroascorbic acid.* This possibility was disproved

Table 2. *Inhibition of urease by ascorbic acid in presence of copper under anaerobic and aerobic conditions*

Test no.		Enzyme preparation used	Time elapsing between addition of Cu and ascorbic acid to enzyme and test of activity (min.)	Cu present (μg./ml. of soln.)	Ascorbic acid initially present (μg./ml. of soln.)	Inhibition due to ascorbic acid as percentage of controls* (%)	Ascorbic acid oxidized (%)
1	Anaerobic	Crystalline	<1	0·1	5	64	2
			30	0·1	5	61	4
			60	0·1	5	62	3
	Aerobic	Crystalline	<1	0·1	5	67	—
			30	0·1	5	62	40
			60	0·1	5	68	60
2	Anaerobic	Dialyzed	2	1	10	100	5
	Aerobic		2	1	10	100	20
3	Aerobic	Dialyzed	10	0·2	20	53	12
			30	0·2	20	50	28
			60	0·2	20	51	50
			90	0·2	20	53	66

* Control solutions of the enzyme were treated in the same way, but without the addition of ascorbic acid.

on finding that when a solution containing dehydroascorbic acid was added to urease in the absence of copper there was no inhibition, even after the dehydroascorbic acid had been left in contact with the enzyme for periods up to 2 hr. before the test of activity (Table 3). In the presence of copper,

Table 3. *Influence of copper and time of standing on the inhibition caused by a solution which initially contained dehydroascorbic acid*

Test no.	Enzyme used	Time elapsing between addition of copper and/or D.H.A.* solution to enzyme and test of activity (min.)	Copper present (μg./ml. of soln.)	D.H.A.* initially present (μg./ml. of soln.)	Inhibition due to D.H.A. as percentage of controls†
1	Crystalline	5	0	10	0
		30	0	10	0
		60	0	10	0
		2	1	10	24
		30	1	10	72
		60	1	10	88
2	Dialyzed	2	0	10	0
		15	0	10	0
		60	0	10	0
		120	0	10	0
		2	1	10	8
		15	1	10	28
		60	1	10	50
		120	1	10	51

* D.H.A. = dehydroascorbic acid.

† Control solutions of the enzyme were similarly treated, but without the addition of the solution of dehydroascorbic acid.

however, inhibition increased with the length of time the enzyme had been previously left in contact with the dehydroascorbic acid. With ascorbic acid under similar conditions the maximum inhibition was attained after a period of less than 1 min. (cf. Table 3). This delay in obtaining maximum inhibition with solutions of dehydroascorbic acid might be attributed to either a slow reaction between the acid and the reactive group of the enzyme, or the slow production of an inhibitor from it. The delay could not be explained by the former hypothesis, for the inhibition was almost as great when the dehydroascorbic acid solution, after standing 1 hr., was added to the enzyme, as when the enzyme was left in contact with the dehydroascorbic acid solution for 1 hr. (Table 4, test 1). These results suggested that dehydroascorbic acid itself, even in the presence of copper, does not inhibit urease, but a product formed from it is responsible for the effect.

Copper was not necessary for the formation of the inhibitor from the dehydroascorbic acid, although it was necessary for the reaction between the inhibitor and the enzyme (Table 4, test 2).

Exp. 4. *Correlation between rate of decomposition of dehydroascorbic acid and development of inhibition.* The conclusions reached in the last section were confirmed by an experiment which established that there was a close parallel between the extent of decomposition of dehydroascorbic acid and the development of inhibitory power.

10 ml. of a solution of dehydroascorbic acid in 0·05 M-phosphate-citric acid buffer, pH 3·5, were added to 90 ml. of a solution containing 50 ml. of phosphate buffer, pH 6·8, to give a final concentration of dehydroascorbic acid of 5 mg./100 ml. and a pH value of 6·4. This solution was allowed to stand at room temperature and at intervals 1 ml. was added to the enzyme solution in phosphate buffer (pH 6·8) containing copper (1 μg./ml.). After 2 min. urea was added and the enzymic activity determined. Samples of the dehydroascorbic acid solution were also analyzed by reduction with H_2S, to determine the rate of decomposition of the dehydroascorbic acid.

The results (Fig. 1) show that a substance is formed from dehydroascorbic acid which, in the presence of copper, causes inactivation of the

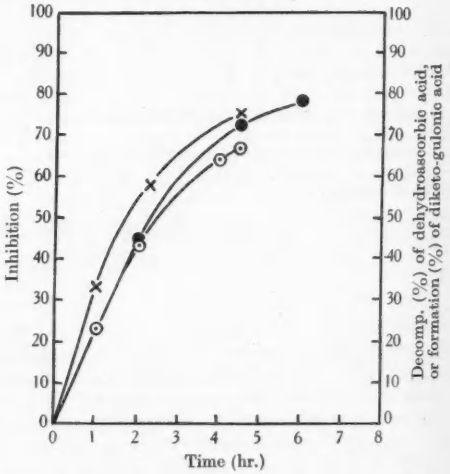

Fig. 1. Correlation between development of inhibition, decomposition of dehydroascorbic acid and formation of 2:3-diketo-gulonic acid. ×—× Inhibition of urease, ⊙—⊙ decomposition of dehydroascorbic acid, •—• formation of diketo-gulonic acid.

enzyme. It was concluded that this substance was 2:3-diketo-*l*-gulonic acid (which is formed from dehydro-*l*-ascorbic acid in aqueous solution by mutarotation), for determinations of 2:3-diketo-

Table 4. *Conditions affecting the development of inhibition by a solution initially containing dehydroascorbic acid*

(*Test no.* 1. To 1 ml. of a solution of dehydroascorbic acid were added 2 ml. of a solution containing copper, 5 ml. of phosphate buffer (pH 6·8) and 1 ml. of a urease solution. Allowed to stand 1 hr. at 20°. Another 8 ml. was allowed to stand 1 hr. before the addition of the urease. The activity of the enzyme in both solutions was then determined. A control was similarly treated with copper, but without dehydroascorbic acid.)

Test no.	Enzyme used	Copper present (μg./ml. of soln.)	D.H.A.* present initially (μg./ml. of soln.)	Treatment	NH_3 produced (μg./ml.)	Inhibition of urease activity (%)
1	Dialyzed	1	10	Enzyme in contact with D.H.A. soln. for 1 hr.	0·10	91·5
		1	10	D.H.A. soln. after standing 1 hr. added to enzyme	0·18	85
		1 (control)	0	—	1·20	—

(*Test no.* 2. Two solutions of dehydroascorbic acid in phosphate buffer were prepared as described above, one containing copper and one without. After standing 1 hr. at 20° urease was added to each of these solutions, and to a further sample of the dehydroascorbic acid solution without copper both urease and copper were added. The activity of the enzyme in each of these solutions was then determined. Controls without dehydroascorbic acid, but with and without added copper, were similarly prepared.)

Test no.	Enzyme used	Copper present (μg./ml. of soln.)	D.H.A. present initially (μg./ml. of soln.)	Treatment	NH_3 produced (μg./ml.)	Inhibition of urease activity (%)
2	Dialyzed	0	10	D.H.A. soln. after standing 1 hr. added to enzyme	1·30	6
		0 (control 1)	0	—	1·38	—
		1	10	D.H.A. soln. after standing 1 hr. added to Cu and enzyme	0·16	87
		1	10	D.H.A. soln., which contained Cu and had stood for 1 hr., added to enzyme	0·20	83·5
		1 (control 2)	0	—	1·21	—

* D.H.A. = dehydroascorbic acid.

gulonic acid in the solution by the method of Penney & Zilva (1943) showed a close correspondence between the production of this acid, the extent of the decomposition of dehydroascorbic acid and the rise in inhibition.

Exp. 5. *Correlation of inhibitory activity with changes occurring in a solution of ascorbic acid during its oxidation in the presence of* Cu^{++}. The results previously recorded indicated that, in the presence of Cu^{++}, inactivation of urease can result from either the addition of ascorbic acid, or its degradation product, 2:3-diketo-gulonic acid, but not of dehydroascorbic acid. Further evidence in favour of this view was obtained by following the changes in inhibitory effect of ascorbic acid undergoing oxidation in the presence of Cu^{++} (Fig. 2). Samples of a solution of ascorbic acid in phosphate buffer (pH 6·8) containing copper, after standing for varying periods of time, were added to urease; after a further period of 2 min. urea was added and the enzymic activity determined. At the start of the experiment the inhibition obtained was nearly complete, but

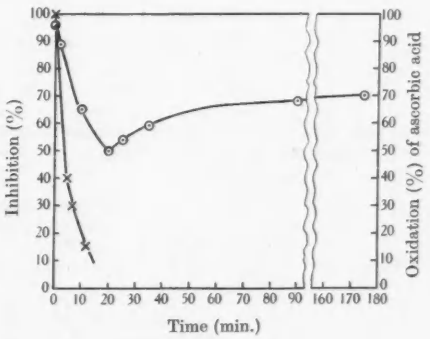

Fig. 2. Correlation of inhibitory activity with changes occurring in a solution of ascorbic acid during its oxidation in presence of Cu^{++}. Urease (dialyzed) was added to a solution of ascorbic acid (10 μg./ml.) in phosphate buffer (pH 6·8) with Cu^{++} (1 μg./ml.) after different time intervals. ⊙—⊙ Inhibition of urease, ×—× oxidation of ascorbic acid.

Table 5. *Inhibition of urease by* 2:3-*diketo-gulonic and* 2-*keto-gulonic acids*

Test no.	Enzyme	Copper present (μg./ml. of soln.)	2:3-Diketo-gulonic acid present (μg./ml. of soln.)	Inhibition of urease activity (%)
1	Dialyzed	0 (control)	0	—
		0	2·5	1
		0	5	4
		0	10	0
		1 (control)	0	—
		1	2·5	58
		1	5	74
		1	10	81
2	Dialyzed	0 (control)	0	—
		0	5	0
		0	10	0
		0	20	0
		1 (control)	0	—
		1	5	70
		1	10	81
		1	20	89
			2-Keto-gulonic acid present (μg./ml. of soln.)	
3	Dialyzed	0 (control)	0	—
		0	5	1·5
		0	10	2
		0	20	3
		0	100	2
		1 (control)	0	—
		1	10	20
		1	20	27
		1	100	37
		0·5 (control)	0	—
		0·5	5	6
		0·5	10	9·5
		0·5	20	15·5
		0·5	100	26

Table 5*a*. *Comparison of effect on urease of ascorbic acid and* 2:3-*diketo-gulonic acid*

Test no.		Copper present (μg./ml. of soln.)	Addition	Concentration (mM)	Inhibition (%)
4	Crystalline enzyme	0 (control)	0	—	—
		0·3	0	—	28
		0·3	Ascorbic acid	0·011	90
		0·3	2:3-Diketo-gulonic acid	0·011	68

In tests 1 and 4, 2:3-diketo-gulonic acid added as Na salt; in test 2, as Ca salt.

as the ascorbic acid was converted to dehydro-ascorbic acid the inhibition first diminished, but subsequently increased. As the previous experiment showed, this increase could be correlated with the formation of 2:3-diketo-gulonic acid.

Exp. 6. *Inhibition by* 2:3-*diketo-gulonic acid and* 2-*keto-gulonic acid.* Confirmation that the oxidation product of ascorbic acid, which caused the inhibition, was 2:3-diketo-gulonic acid was obtained in experiments in which pure preparations of this acid were used (Table 5). Diketo-gulonic acid only acts as an inhibitor in the presence of copper but is less effective than ascorbic acid in an equivalent amount (Table 5*a*). With the related compound, 2-keto-gulonic acid, there was appreciable inhibition only in relatively high concentration.

Exp. 7. *Role of copper, and comparison of inhibition by* Cu^{++} *and* Cu^{+}. Since ascorbic acid readily reduces $Cu^{++} \rightarrow Cu^{+}$ it seemed worth while to ascertain how far the inhibitory action of ascorbic acid could be correlated with this reaction. The effect of equimolecular amounts of Cu^{++} and Cu^{+} salts was, therefore, examined.

All solutions were first freed of O_2 by the passage of O_2-free N_2. Stock solutions of $CuCl_2$ and CuCl were prepared by dissolving the required amount of the salts in 2N-HCl. To a solution containing 5 ml. of phosphate buffer (pH 6·8) and 1 ml. of enzyme solution, made up with water to a volume of 8·9 ml., 0·1 ml. of either the Cu^{++} or Cu^{+} solution was added. Parallel experiments were also set up in which ascorbic acid was present. To the control solutions 0·1 ml. of 2N-HCl was added in place of the copper solution. The enzyme was left in contact with the Cu^{+} or Cu^{++} and ascorbic acid for 1 min. before the addition of urea. The reaction period in these experiments was 5 min. O_2-free N_2 was passed through the solution throughout the period of test.

The results (Table 6) indicate (1) that Cu^{+} ions, in equivalent concentration, have a much greater

Table 6. *Comparison of effect of* Cu^{++} *and* Cu^{+} *on urease*

Test no.	Enzyme	Cu^{++} present (μg./ml. of soln.)	Cu^{+} present (μg./ml. of soln.)	Ascorbic acid present (μg./ml. of soln.)	Inhibition of urease activity (%)
1	Dialyzed	0·5	—	—	35
		0·5	—	5	95
		0·5	—	10	97
		—	0·5	—	89
2	Dialyzed	0·2	—	—	7
		0·2	—	10	61
		—	0·2	—	48
3	Dialyzed	0·5	—	—	34
		0·5	—	5	88
		0·5	—	10	88
		—	0·5	—	91
4	Crystalline	0·5	—	—	50
		—	0·5	—	100
		0·2	—	—	25
		—	0·2	—	94
5	Crystalline	0·2	—	—	20
		0·2	—	5	98
		0·2	—	10	98
		—	0·2	—	97

inhibitory action on urease than Cu^{++} ions, and (2) that the maximum inhibition obtained by the addition of ascorbic acid to urease solutions containing Cu^{++} was identical with that obtained with Cu^{+} alone. Experiments of a similar nature (Fig. 3)

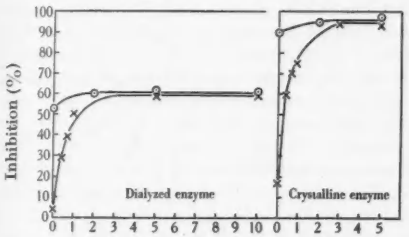

Fig. 3. Effect of ascorbic acid in varying concentration on inhibition of urease by Cu^{++} or Cu^{+} (concentration of Cu 0·2 μg./ml.). ⊙—⊙ Cu^{+}, ×—× Cu^{++}.

also show that ascorbic acid has no additional inhibitory influence when added to a solution containing urease and a cuprous salt.

These facts are most simply explained on the assumption that the inhibitory action of ascorbic acid in the presence of Cu^{++} is due to the reduction of $Cu^{++} \rightarrow Cu^{+}$, with a consequent increase in inhibitory power.

Exp. 8. *Protection and reactivation of urease by thiol compounds.* Both cysteine and glutathione protected the enzyme against inactivation by either Cu^{++} plus ascorbic acid, or by Cu^{+} alone (Table 7*a*). This effect is no doubt due to a reaction between the copper salt and thiol group, whereby the —SH group of the enzyme is protected.

Hellerman, Perkins & Clark (1933) showed that urease inactivated by Cu_2O could be reactivated by treatment with H_2S. We have found that the inactivation of urease, either by Cu^{+} or by Cu^{++} plus ascorbic acid, can be reversed by H_2S.

Solutions of the enzyme in phosphate buffer (pH 6·8) were first freed of O_2 and the enzyme inactivated by the addition of either $CuCl_2$ plus ascorbic acid, or CuCl. After standing 2 min. at 20°, H_2S was passed through a sample of the inactivated enzyme solution for 5 min. Urea was added at once and the enzymic activity determined. A control solution was treated with H_2S: there was no increase in the activity as a result of the H_2S treatment.

The results show that complete reactivation in both cases was obtained. Cysteine in place of H_2S gave a similar result (Table 7*b*).

Exp. 9. *Influence of other metallic salts.* The effect on urease of ascorbic acid in the presence of small amounts of Ag^{+}, Au^{+++}, Hg^{++} or Fe^{+++} ions is shown in Table 8. Except in the case of Au^{+++} the addition of ascorbic acid caused little or no increase in the degree of inhibition. Although the inhibition of the enzyme by Au^{+++} alone was equal to that caused by Cu^{++} (cf. Table 1, tests 2 and 3), the increase in the inhibition on addition of ascorbic acid was less than the corresponding effect with Cu^{++}. The toxicity of Hg^{++} was reduced by the addition of ascorbic acid. In equivalent concentrations Hg^{++} inhibited more than Hg^{+} (Table 8*a*); the reduced toxicity of Hg^{++} salts in the presence of ascorbic acid was presumably due to the reduction of $Hg^{++} \rightarrow Hg^{+}$. Other metallic salts were tested, and the results showed that in solutions containing Zn^{++}, Sn^{++++}, Pb^{+} or Co^{++} in a concentration of 0·016 mM., ascorbic acid had no inhibitory action on urease.

Table 7*a*. *Protection of urease by thiol compounds*

Enzyme	Cu^{++} present (μg./ml. of soln.)	Ascorbic acid present (μg./ml. of soln.)	Cu^{+} present (μg./ml. of soln.)	Cysteine present (mM)	Glutathione present (mM)	Inhibition (%)
Crystalline	0·5	3	—	—	—	100
	0·5	3	—	1	—	5
	0·5	3	—	—	1	0
	—	—	0·5	—	—	100
	—	—	0·5	1	—	0
	—	—	0·5	—	1	0

Table 7*b*. *Reactivation of urease by thiol compounds*

Enzyme	Cu^{++} present (μg./ml. of soln.)	Ascorbic acid present (μg./ml. of soln.)	Cu^{+} present (μg./ml. of soln.)	Inhibition (%)	Reactivation. Inhibition (%) after treatment with	
					H_2S	Cysteine ($M \times 10^{-3}$)
Crystalline	0·2	3	—	99	0	—
	—	—	0·2	97	0	—
	0·5	3	—	100	—	4
	—	—	0·5	100	—	6

Table 8. *Effect of ascorbic acid on the inhibition of urease in the presence of* Ag^+, Au^{+++}, Hg^{++}, *or* Fe^{+++}

(In these experiments a dialyzed preparation of urease was used.)

Salt	Concentration (mM)	Ascorbic acid present (μg./ml. of soln.)	Inhibition of urease activity (%)
$AgNO_3$	0·00475	0	54
		5	62
		10	62
		20	57
$AgNO_3$	0·0032	0	47
		20	54
$HAuCl_4$	0·00475	0	24
		5	45
		10	51
		20	55
$HAuCl_4$	0·0009	0	5
		5	7
		10	8
		20	8
$Hg(CH_3COO)_2$	0·0064	0	65
		5	56
		10	43
		20	36
$Hg(CH_3COO)_2$	0·0032	0	33
		5	26
		10	10
$Hg(CH_3COO)_2$	0·0016	0	15
		20	0
$FeCl_3$	0·016	0	11
		10	16
$FeCl_3$	0·016	0	2
		10	0

Table 8*a*. *Comparison of effect of* Hg^{++} *and* Hg^+ *on urease*

$Hg(CH_3COO)_2$	0·0032	0	36
$Hg(CH_3COO)$	0·0032	0	7
$Hg(CH_3COO)_2$	0·0126	0	92
$Hg(CH_3COO)$	0·0126	0	63

Exp. 10. *Tests with other substances.* Hydroxytetronic, reductic and dihydroxymaleic acids, which all contain a dienol group, increased the inhibitory effect of Cu^{++} on urease to a degree comparable with that observed with ascorbic acid. Adrenalin behaved likewise, though the inhibition was much less marked than with ascorbic acid.

Quinone, in contrast to these substances, inhibited in the complete absence of Cu^{++}, for its action was not reduced when substances such as pyrophosphate were present. The inhibition in this case is no doubt due to a reaction between quinone and an essential —SH group of the enzyme (cf. Quastel, 1932).

DISCUSSION

There has been a general consensus of opinion that the inhibitory action of many different substances on urease can be most simply explained on the assumption that they react as oxidants, or that they form derivatives with at least one of the enzyme's —SH groups, the integrity of which is essential for activity (Hellerman, 1937, 1939; Hellerman, Chinard & Dietz, 1943).

Hopkins (1929) showed that Cu_2O converts mercaptans (RSH) to the corresponding mercaptides (RSCu). With glutathione he found that the reaction could be initiated by the addition of a cupric salt, the Cu^{++} ion being first reduced to Cu^+ by the thiol group, and the resulting Cu^+ ion reacting to form the insoluble copper derivative of glutathione. Hellerman *et al.* (1933) noted the inhibitory action of Cu_2O on the activity of urease, which was attributed to the formation of a cuprous mercaptide. They also showed that Cu^{++} salts did not rapidly inactivate urease; only after aeration of the solution for 2–3 hr. was the enzymic activity destroyed. They considered the toxic effect of the Cu^{++} was due not to the ion *per se*, but to its ability to accelerate catalytically the oxygenation of urease.

The experiments reported in this paper have emphasized the comparative non-toxicity of Cu^{++} compared with Cu^+ ions. The simplest explanation of the toxic action of ascorbic acid on urease is that it is able to reduce $Cu^{++} \rightarrow Cu^+$. It was expected, therefore, that other compounds containing the dienol group, e.g. reductic, hydroxytetronic and dihydroxymaleic acids would exert a similar effect, and this has indeed been found to be so. Of interest too is the fact that adrenalin mildly inhibits urease in the presence of Cu, but not in its absence.

The inhibitory action of 2:3-diketo-gulonic acid is probably due to its ability to reduce Cu^{++}. In fact this substance acts as a reducing agent at pH values above 4·0 (Borsook, Davenport, Jeffreys & Warner, 1937), and it reduces methylene blue in the presence of phosphate (Frankenthal, 1944). It is conceivable that the reductant in such solutions is the enolized form of the keto acid.

Whether ascorbic acid acts as an inhibitor of urease activity *in vivo* is more doubtful. The present work has emphasized that Cu^{++} ions must be present if ascorbic acid is to act as an inhibitor. Whether the mechanism of the inhibitory action of ascorbic acid on plant β-amylase (Purr, 1934; Seshagiri Rao & Giri, 1942), which is reported to require an —SH group for its activity, is the same as with urease remains to be determined. It is, however, suggestive that the latter workers found that the action of ascorbic acid was increased in the presence of copper.

It seems unlikely that the inhibition of such enzyme systems can be considered as an essential role of ascorbic acid in the cell, in view of the well-marked inhibitory properties of other dienols which are biologically inactive.

SUMMARY

1. Evidence is presented which shows that Cu^{+} is a much more effective agent than Cu^{++} in inhibiting urease, and that ascorbic acid acts as an inhibitor only in so far as it reduces $Cu^{++} \rightarrow Cu^{+}$. The experimental findings on which this evidence is based are as follows:

(*a*) Ascorbic acid does not inhibit urease when copper is absent, but does so when copper is present.

(*b*) The inhibition by Cu^{++} plus ascorbic acid is of the same magnitude as with Cu^{+} alone. The inhibition produced by Cu^{+} is not increased by the addition of ascorbic acid.

(*c*) Other reducing substances containing a dienol group and capable of reducing $Cu^{++} \rightarrow Cu^{+}$, e.g. hydroxytetronic, reductic and dihydroxymaleic acids, resemble ascorbic acid in their power to inactivate urease in the presence of copper.

(*d*) Urease inactivated either by Cu^{++} plus ascorbic acid or by Cu^{+} alone may be reactivated by H_2S, or by cysteine. It is postulated that the Cu^{+} inactivates by forming a derivative with an —SH group of the enzyme, and that substances like H_2S reactivate by removing the bound copper and regenerating this —SH group.

2. Dehydroascorbic acid does not inhibit urease, even in the presence of copper. 2:3-Diketo-gulonic acid (produced by mutarotation when solutions of dehydroascorbic acid are left standing) inhibits only in the presence of copper, but to a less extent than does ascorbic acid.

3. Of the metallic salts tested, Cu^{++} and Au^{+++} alone had their toxicity increased by the addition of ascorbic acid. With Hg^{++} salts the toxicity was reduced by ascorbic acid.

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Blood Haemoglobin: the Relationship between Oxygen Capacity and Iron Content of Blood in Men and Women

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In a recent paper (Gibson & Harrison, 1945) we described the preparation of a stable artificial standard solution which matched the absorption spectrum of blood in 0·1 N-NaOH over a considerable range of the visible spectrum. In order to assign a haemoglobin value to this solution for use in the estimation of blood haemoglobin, we standardized it against samples of normal male blood of known oxygen capacity and iron content. It was found that the haemoglobin values of the blood samples determined by oxygen capacity measurement were slightly, but consistently, lower than those obtained by iron estimation, a finding which agrees with results which have been obtained by others, for example, Macfarlane & O'Brien (1944). The latter workers, however, obtained results with blood samples from women which suggested that in them this relationship is reversed, the haemoglobin value derived from oxygen being higher than that from iron. They pointed out, however, that this result was unexpected and that their investigation had not been planned to determine the extent of any such sex difference.

It seemed quite possible that if there were a systematic sex difference in the iron:oxygen ratio, there might also be a sex difference in the iron:colour

and oxygen: colour ratios, and that different haemoglobin equivalents might be required for the artificial standard with blood from the two sexes. We therefore decided to carry out two parallel series of determinations of oxygen capacity and iron content on the blood of normal men and women, taking every possible precaution to eliminate any systematic error affecting the sexes unequally.

The results would serve both to check the figures given for the standard for male blood and to establish a value for female blood if, in fact, a sex difference were found.

METHODS

A detailed description of the methods used has already been published (Gibson & Harrison, 1945), and only a brief outline will be given here. The same calibrated apparatus was used as in the above paper.

Blood samples. 30 ml. venous samples were taken from normal male and female medical students about 2–3 hr. after breakfast. A stainless steel needle and an all-glass syringe were used and coagulation was prevented by 1 mg./ml. of blood of a 2:3 mixture of potassium and ammonium oxalates. A male sample and a female sample were taken within a few minutes of one another and the two specimens were carried through all the analytical processes side by side. On alternate days the time sequence of collecting the bloods and of carrying out the subsequent processes was reversed as between the sexes, and where different apparatus was used for the two samples a similar day by day alternation was maintained.

Oxygen capacity. The determinations were carried out in duplicate with the constant-volume Van Slyke-Neill manometric apparatus. They were usually completed within 2 hr. of taking the blood samples. The values included any carboxyhaemoglobin present. The average deviation of a determination from the mean was 0·03 g. of haemoglobin/100 ml. of blood.

Methaemoglobin. This pigment was determined photoelectrically and, where necessary, a correction was made to the observed oxygen capacity value. The figure for methaemoglobin never exceeded 0·1 g./100 ml. and was usually negligible.

Iron in blood. Iron was determined in triplicate on 3 ml. samples by the modification of the method of Klumpp (1934) previously described. The average deviation of a determination from the mean was 0·2% of the amount measured. A fresh iron standard was made from electrolytic iron (purity greater than 99·9%) and was checked with standard $K_2Cr_2O_7$. From this a dilute standard of ferric iron was prepared after oxidation with H_2O_2. This standard was compared by $TiCl_3$ titration with a fresh dilute standard made from the strong iron standard used in our previous work. Exact agreement between the two solutions was obtained.

Measurements in the absorptiometer. The blood was diluted 1 in 101 with 0·1 N-NaOH and after mixing for a few minutes, 10 ml. of the solution were heated in a stoppered boiling tube in a boiling water-bath for exactly 4 min., a similar amount of the artificial standard being heated in the same way. After being rapidly cooled, the samples were compared in turn in the same 1 cm. glass cell against water in a Spekker absorptiometer using Hilger-Chance OGr.1 green filters. A neutral grey screen of about 0·5 density was measured immediately after each sample in order to control any chance fluctuations in the absorptiometer readings.

RESULTS

The haemoglobin values obtained from oxygen capacity and from iron content for ten men and ten women are given separately in Table 1. (The

Table 1. *Blood haemoglobin of men and women, and haemoglobin equivalents of artificial standard based on oxygen capacity and iron content*

(The figures have been calculated on the assumption that 1 g. haemoglobin combines with 1·34 ml. of oxygen and contains 0·334% Fe. The haemoglobin equivalents of the standard were derived by comparison with blood diluted 1 in 101 with 0·1 N-NaOH (Gibson & Harrison, 1945).)

	Males				Females			
	Haemoglobin in blood from		Haemoglobin equivalent of standard from		Haemoglobin in blood from		Haemoglobin equivalent of standard from	
	O_2 (g./100 ml.)	Fe (g./100 ml.)	O_2 (g./100 ml.)	Fe (g./100 ml.)	O_2 (g./100 ml.)	Fe (g./100 ml.)	O_2 (g./100 ml.)	Fe (g./100 ml.)
	15·35	15·78	15·64	16·15	13·88	14·39	15·79	16·44
	16·67	16·94	15·88	16·27	13·62	13·78	15·84	16·13
	15·07	15·54	15·60	16·05	15·57	15·64	16·16	16·24
	16·09	16·48	16·15	16·50	16·21	16·67	15·95	16·36
	16·38	16·55	15·80	15·96	13·91	14·26	15·60	16·00
	14·76	15·09	15·68	16·06	15·55	15·85	15·68	16·01
	15·79	15·96	15·95	16·13	14·24	14·49	15·61	15·90
	16·94	17·35	15·60	16·01	15·39	15·57	15·72	15·93
	15·47	15·59	15·83	15·96	14·95	15·20	15·81	16·06
	14·65	14·96	15·59	15·95	15·06	15·41	15·82	16·18
Mean:	15·72	16·02	15·77	16·10	14·84	15·13	15·80	16·13
V%*:	—	—	1·2	1·1	—	—	1·1	1·1

* V = coefficient of variation.

figures in the second decimal place are only approximate but have been retained in order to allow the best possible estimate of the O_2:Fe ratio.) The haemoglobin equivalent of the artificial standard based on oxygen capacity and iron determinations is given for each blood in the remaining columns.

Our results indicate that there is no difference in the O_2:Fe ratio between men and women. The numbers examined were not large, but the average of the individual O_2:Fe ratios was identical in each sex. The mean value corresponded to 393 ml. O_2/g. Fe, the values ranging from 388 to 398 ml. in men and 386 to 399 ml. in women. The coefficients of variation were 0·8 % in men and 0·9 % in women. (The plasma Fe content is too small to affect the above results and has been neglected.)

The values for the artificial standard agree well with those derived from ten male bloods in our previous paper, the present value based on oxygen capacity being 15·78 as against 15·60 g. of haemoglobin/100 ml., and the present value from iron being 16·11 as against 16·01 found previously. The average values for the standard, based on determinations on all thirty bloods are 15·7 g. from oxygen and 16·1 g. of haemoglobin/100 ml. from Fe. We have found that there is no difference between the sexes with regard to the ratio between iron and colour and oxygen and colour, so that the same haemoglobin equivalent may be used for the standard in haemoglobin determinations carried out on the blood from persons of either sex.

To estimate the mean blood haemoglobin level of the medical students who assisted us by acting as donors in our work, the values for the ten men who were tested in the work already published (Gibson & Harrison, 1945) were combined with those for the ten men tested on this occasion. Assuming an equivalent of 14·75 g. Hb/100 ml. for the British Standards Institution Haldane standard (Macfarlane & O'Brien, 1944; King, Gilchrist & Matheson, 1944), and using our oxygen capacity figures for the bloods, the mean Haldane value for the twenty men students was 107 (S.D. (standard deviation) 7). The blood samples were taken in 1945, and the value is almost the same as that found for fifty-two men students in 1943 in the haemoglobin survey carried out in England by the Medical Research Council Committee on Haemoglobin Surveys (1945). The average in the English survey was 106·4 (S.D. 6·4) on the British Standards Institute Haldane scale. The corresponding value for our small series of ten women medical students was 101 (S.D. 6) as compared with 92·8 (S.D. 6·8) from fifty-nine women students in the English survey.

SUMMARY

1. The ratio between oxygen capacity and Fe content is the same in the blood of men and women. The specific oxygen capacity found was 393 ml. O_2/g. Fe (standard error of mean 0·8 ml.) compared with the stoichiometric value of 401 ml.

2. The artificial standard may be used with the same values for blood from either sex.

3. Values for the artificial standard agree closely with those previously published, the mean values from thirty bloods being 15·7 g. of haemoglobin/100 ml. of blood from oxygen capacity, and 16·1 g. from Fe.

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Micro-method for the Determination of Vitamin A in Liver Biopsies in Man and Larger Animals

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Since the liver contains over 95 % of the vitamin A stored in the organism several problems concerning the metabolism of vitamin A have been elucidated by means of analyses of liver tissue—mainly in small animals, large numbers of which can be used in experiments (With, 1940, 1942). For economical reasons experiments in which animals are killed have been few on larger animals, such as ox, horse, sheep, and swine, and many problems still remain. In man, the storage of vitamin A in the liver has not been seriously studied, although investigations might be possible by means of operation biopsies

combined with analyses of necropsy material. If it were possible to determine the vitamin A content of the minute biopsies taken by means of liver puncture (Iversen & Roholm, 1939), such investigations could be made and, moreover, the vitamin A content of the liver followed at short intervals.

Since the weight of the liver can be regarded as constant during short periods, without committing errors of importance the concentration of vitamin A in the biopsy can be reckoned to be proportional to the vitamin A content of the liver. It is important that the biopsy be taken under standard conditions—for instance, fasting in the morning—in order to exclude errors due to variation in the water content and other possible rhythmical diurnal variations in the composition of the liver.

In this paper is described a method for the estimation of vitamin A in biopsies of liver and a comparison of the results which it yielded with those obtained on autopsy material.

METHODS

By reading the Carr-Price reaction in special small cuvettes ('Kleinkyvetten') it is possible to obtain sufficiently thick layers to get measurable extinctions with the minute amounts of liver tissue of the micro-biopsies. According to Roholm, Krarup & Iversen (1942) such biopsies obtained by the method of Iversen & Roholm (1939) are cylinders of 2×15 mm., weighing about 50 mg. As the vitamin A content of human liver normally is 25–1000 i.u./g. (With, 1940, 1941*b*), it is now possible to calculate the Carr-Price extinction of the vitamin A in such liver biopsies.

If a liver contains 25 i.u./g. a biopsy of 50 mg. contains 1·25 i.u., which after extraction are dissolved in 2·5 ml. of chloroform. If the Carr-Price reaction is performed with the least possible dilution, i.e. equal amounts of solution and reagent, the 1·25 i.u. are dissolved in 5 ml. of reaction mixture, and this is enough for two readings in 5 cm. Kleinkyvetten. If the liver reserves are greater, the unsaponifiable fraction of the biopsy is dissolved in 5–10 ml. of chloroform, and greater amounts of reagent may be used, e.g. 0·2 ml. solution and 0·5 ml. of reagent, 0·1 + 1·0 ml., or 0·02 + 1·0 ml.

Since a surplus of reagent is required smaller amounts of reagent than the volume of the test solution give too low extinctions, and it is only in the case of weak solutions that equal volumes are sufficient to give quantitative reaction. Measurements on solutions containing 0·5–2 i.u./ml. of vitamin A showed with an equal volume of reagent the same extinctions as with greater volumes, provided that the extinctions were calculated for the same volume.

From measurements of the Carr-Price reaction and ultraviolet spectral absorption of liver extracts, the extinction value of 1·0 in the Carr-Price reaction—measured with the filter S61 of the Pulfrich photometer in a 1 cm. thick layer—corresponds to a vitamin A content of *c.* 600 i.u./100 ml. of the reaction mixture (Engel, Granström, Lindgren & Norlander, 1942; Jensen & With, 1939; With, 1940). Thus 1·25 i.u. dissolved in 5 ml. must give an extinction of about 0·21 in a 5 cm. thick layer according to Beer's law.

It is possible to read the extinction of the Carr-Price reaction of the vitamin A in aspiration biopsies of the liver even if its vitamin A concentration lies at the lower limit of the normal. In this case, however, the analytical error is considerable, since only two readings of the Carr-Price reaction are possible, but if the liver reserve is greater the analysis can be carried out with an error of $\pm 5\%$ (With, 1940), and this will most often be the case in man and larger animals.

Procedure

If necessary the biopsy specimen is cleansed of adhering blood with a bit of filter paper. A small piece of the specimen may be taken for histological examination to ascertain that it really is liver tissue. Then the specimen is weighed and, if the analysis cannot be made immediately, is placed in a small vessel filled with N_2 or CO_2.

The saponification and extraction, etc., are carried out as previously described (With, 1940). The biopsy specimen is heated carefully with 3 ml. of 5% ethanolic KOH in a little flask which is shaken for a few minutes to produce solution of the tissue. The heating must be as short as possible and 'roasting' has to be avoided. Then the contents of the flask as well as the distilled water with which it is washed are placed in a 50 ml. separatory funnel and extracted three times with 10 ml. of ether free from peroxides. The combined ethereal extracts are washed in the usual way and dried by suction through a firmly compressed layer (*c.* 1 cm. thick) of anhydrous Na_2SO_4 upon a sintered glass filter (e.g. 3 G3); the sulphate is washed twice with 5 ml. of dry ether. The dry ether is then quickly evaporated in a small flask in the presence of N_2 or CO_2 or *in vacuo*; the heating must be mild and short. The residue is dissolved in 1 ml. of freshly distilled chloroform (A.R.), transferred to a 10 ml. measuring cylinder with glass stopper; the flask is washed three times with 0·5 ml. of chloroform and these washings added to the cylinder and the volume adjusted to 2·5 ml. The chloroform solution is used for the determination of vitamin A by the Carr-Price reaction; it may stand several hours in a dark ice-box before the measurement, without loss of vitamin A (Fuchs & Soos, 1943).

Colorimetric measurements are made with the Pulfrich photometer equipped with Kleinkyvetten and filter S61 (With, 1940). At first an orientating experiment is carried out on 0·1 ml. of solution and 0·1 ml. of reagent in a test tube. If this gives a pale-blue colour the reaction is carried out in a 5 cm. Kleinkyvette with 1·25 ml. solution and 1·25 ml. reagent, but if the colour is more intense, 0·1 ml. solution and 1·0 ml. reagent are used—or 0·02 + 1·0 ml., etc.—and 2, 1, or 0·5 cm. Kleinkyvetten according to the intensity of the colour. The 5, 2, 1 and 0·5 cm. Kleinkyvetten require respectively 2·5, 1·0, 0·5 and 0·25 ml. of fluid. If the orientating reaction shows turbidity, one or two drops of acetic anhydride are placed in the bottom of the cuvette before the reading. The control cuvette is filled with chloroform.

The amounts of reagent and solution and the layer of thickness are selected so that the extinction lies between

0·3 and 0·8. The average of four readings of separate mixtures of solution and reagent gives the final result. The first reading—within 5 sec.—of each mixture is used. To obtain rapid and complete mixing of test solution and reagent in the narrow Kleinkyvetten it is essential to blow gently through the reagent pipette, which has to reach close to the bottom of the cuvette.

Measurement of the carotenoids is unnecessary as they are present only in negligible amounts in human liver (With, 1940).

The calculation is then made with the following formula:

$$E^{1\%}_{1\,cm.}\,(S61) = \frac{1000 \times E \times (S+R)}{L \times S \times B \times 40},$$

in which B (in mg.) is the weight of the biopsy, S (in ml.) the volume of the solution, R (in ml.) that of the reagent, L (in cm.) the thickness of layer, and E the extinction reading. $E^{1\%}_{1\,cm.}$ is the extinction of the unsaponifiable residue of 1 g. of liver tissue dissolved in 100 ml. of solution-reagent mixture and measured in a 1 cm. thick layer. The formula is valid only if the unsaponifiable residue of the biopsy is dissolved in 2·5 ml. of chloroform. The concentration in i.u./g. of liver is calculated by multiplying $E^{1\%}_{1\,cm.}$ by 600, mentioned above (With, 1940, 1941*a*).

The photometer used was calibrated by measurements of the standard vitamin A preparation 'Vogan' (Merck) (cf. Willstaedt & With, 1938) and further controlled by comparison of the Carr-Price readings with spectrographic measurements of the 328 mμ extinction in a Hilger quartz spectrograph with echelon cells (Jensen & With, 1939; With, 1940).

RESULTS

Comparison between the micro-method and a macro-method

As the vitamin A concentration of the liver varies (Lindqvist, 1938, p. 240) we examined to what extent arbitrary samples provide values which can be regarded as representative of the average concentration of vitamin A in the liver. Samples were taken from 20 necropsies: specimens of 30–50 mg. from places from which puncture biopsies may originate, and 10–20 specimens of about 10 g. of tissue from different parts of the liver. The results of the analysis of these specimens are seen from Table 1.

The differences between the results of the micro-analyses of specimens from the right lobe from the data from macro-analyses are all below ± 11 %, and their average difference is 4·65 %. The samples from the left lobe show, however, deviations up to 31·3 %. These findings agree with those of Lindqvist (1938). Since the left lobe constitutes about 20 % of the liver the errors committed by reckoning a micro-sample from the right lobe as indicating the average for the whole liver are small, and without committing significant errors one may reckon the deviation of biopsy micro-analyses from the average vitamin A concentration of the liver to be *c.* 5 %, with *c.* 12 % as maximal value.

Table 1. *Comparison between the results of macro-analyses and those of micro-analyses*

(*d* indicates samples from right lobe of liver; *s*, samples from left lobe.)

	$E^{1\%}_{1\,cm.}$ from		Difference (results of micro-analysis minus result of macro-analysis) expressed as percentage of result of macro-analysis
	Macro-analysis	Micro-analysis	
1	0·057	0·063 (*d*)	+10·5
2	0·492	0·462 (*d*)	− 6·1
		0·540 (*d*)	+ 9·8
3	0·420	0·423 (*d*)	+ 0·7
		0·401 (*d*)	− 4·5
4	0·107	0·112 (*d*)	+ 4·7
		0·090 (*s*)	−15·9
5	0·384	0·392 (*d*)	+ 2·1
		0·264 (*s*)	−31·3
6	0·038	0·040 (*d*)	+ 5·3
		0·037 (*s*)	− 2·6
7	0·360	0·352 (*d*)	− 2·2
8	0·474	0·463 (*d*)	− 2·3
9	0·990	1·07 (*d*)	+ 8·1
10	1·32	1·28 (*d*)	− 3·0
		0·96 (*s*)	−27·3
11	0·114	0·107 (*d*)	− 6·3
		0·135 (*s*)	+18·4
12	0·260	0·271 (*d*)	+ 4·2
13	0·569	0·538 (*d*)	− 5·4
14	0·617	0·599 (*d*)	− 2·9
		0·505 (*s*)	−18·2
15	0·742	0·769 (*d*)	+ 3·6
		0·710 (*s*)	− 4·3
16	0·323	0·327 (*d*)	+ 1·2
17	0·076	0·083 (*d*)	+ 9·2
18	0·115	0·108 (*d*)	− 4·9
19	0·249	0·238 (*d*)	− 4·4
		0·203 (*s*)	−18·5
20	0·466	0·472 (*d*)	+ 1·3
		0·484 (*s*)	− 3·7
Mean	0·408	0·413 (*d*)	+ 0·85 ± 5·5 (*d*)
		0·376 (*s*)	−10·60 ± 16·3 (*s*)

While the advantage of the puncture biopsy is that it can be repeated, its main disadvantage is its greater margin of error (cf. Table 1). Consequently the accuracy of an estimation of the liver depots based on a puncture biopsy must be inferior to that of one based on an operation biopsy, and especially to one based on necropsy material.

SUMMARY

A method is described for the quantitative determination of vitamin A in aspiration biopsies of the liver by the Carr-Price reaction. It provides a means of studying the storage in the vitamin A and carotenoids in man and larger animals on a much greater scale than before.

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The Application of the Silica Gel Partition Chromatogram to the Estimation of Volatile Fatty Acids

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The lower fatty acids, with the exception of formic acid, are so similar in their chemical properties that their quantitative separation and estimation by purely chemical methods have not proved possible. On the other hand, marked changes in physical properties occur as the series is ascended; for example, the volatility in steam increases and the partition coefficient between water and immiscible organic solvents decreases. It is not surprising, therefore, that the methods available for the estimation of these compounds in mixtures should be physical rather than chemical.

The distillation procedure of Duclaux (1874) and its numerous refinements make use of the observation that the volatile fatty acids distil in steam at different rates, and that in a mixture each distils at its own characteristic speed regardless of the presence of others. The partition method introduced by Behrens (1926), subsequently improved by Werkman (1930), depends on the fact that each fatty acid partitions itself between water and an immiscible organic solvent irrespective of the other acids present. Although both these methods, in the absence of more satisfactory ones, have found favour with the analyst, it should be emphasized that both have their limitations. They are inadequate for work with more than two acids, they are of little use by themselves for the identification of the constituents of a mixture, and neither achieves a complete resolution of even a binary mixture into its components. In consequence, the amount of each acid present is obtained by calculation rather than by direct assay. Indeed, until recently, no method has been available for the separation, identification and estimation of the lower fatty acids in a mixture. Schicktanz, Steele & Blaisdell (1940) introduced a procedure based on the fractional distillation, with an efficient column, of the azeotropes formed between the lower fatty acids and aryl hydrocarbons. In this manner they were able to separate a mixture containing formic, acetic, propionic, *n*-butyric and *iso*-butyric acids into its components. This represented a notable advance in technique, but the method is not quantitative and is scarcely suitable for routine analysis.

Lester Smith (1942) demonstrated that the silica-gel partition chromatogram, developed by Martin & Synge (1941), would separate a mixture of formic, acetic, propionic, *n*-butyric and *n*-valeric acids in chloroform into its component acids. Only compounds with differing partition coefficients between water and the organic developing solvent can be separated by this method. Martin & Synge (1941) incorporated in the gel an indicator of suitable pK for the location of colourless organic acids on the column, and for the separation of acetylated amino-acids they initially recommended methyl orange; this indicator was subsequently supplemented by anthocyanins (Gordon, Martin & Synge, 1943, 1944) which are not leached so readily as methyl orange. More recently Liddell & Rydon (1944) have introduced an azo dye, 3:6-disulpho-β-naphthalene-azo-*N*-phenyl-α-naphthylamine, with similar properties and the added advantage that it is readily prepared in quantity in the laboratory. Lester Smith (1942) found that methyl orange was of little use when the partition chromatogram was used for the separation of lower fatty acids, owing to lack of sensitivity, the

volatile fatty acids having a significantly higher pK than the acetylated amino-acids, and he recommended bromocresol green (pK 4·66) as a suitable alternative. This has been confirmed, and in addition it has been found that both anthocyanins (pelargonin) and the Liddell & Rydon indicator are insensitive as compared with bromocresol green.

Lester Smith published no investigation of the quantitative aspects of the method, but the procedure was of value for the identification of lower fatty acids present in a chloroform solution; furthermore the results appeared so promising that a further investigation has been made with the object of developing a routine method for the separation, identification and quantitative estimation of volatile fatty acids in biological materials, and particularly in fermentation fluids.

EXPERIMENTAL

Preparation of the chromatogram

Silica gels prepared by the method of Gordon *et al.* (1943) gave the most suitable chromatograms when 3 g. of gel were mixed with 1·8 ml. of water; such a preparation, however, was acid to bromocresol green, and remained so after washing with large volumes of distilled water. It was found expedient therefore to add a quantity of 0·1 N-NaOH to the bromocresol green solution such that 1·8 ml. of the indicator mixed with 3 g. of gel gave a light blue powder. A solution of bromocresol green (0·2%), made according to Cole (1933), and containing in addition 17·4 ml. of 0·1 N-NaOH/100 ml., was employed. This quantity of NaOH has been found adequate for all preparations of gel so far made.

The developing solvents used were a mixture of chloroform and butanol. Technical chloroform was purified by washing thoroughly with tap water to remove ethanol, drying over $CaCl_2$, and distilling over a packed column, the fraction boiling at 61–62° being collected. Used chloroform was recovered in the same way after a preliminary extraction with NaOH solution to remove acid substances. Two chloroform-butanol mixtures were employed containing 1 and 5% (v/v) of butanol respectively (CB_1 and CB_5). They were stored dry in brown glass bottles; prior to use on the chromatogram they were saturated with distilled water.

The chromatogram tubes were about 40 cm. in length and constricted at one end. A perforated silver disk, bearing a circle of filter paper, was lodged on the constriction and this served to support the column. The gel (3 g.) was weighed, transferred to a clean mortar, and ground to a fine powder. The indicator solution (1·8 ml.) was added and the mixture ground with the aid of a pestle and a stainless steel spatula until a homogeneous, finely divided, light blue powder was obtained. A small amount of wet CB_1 was added and the resulting green paste ground for a further period; more wet CB_1 was added, to a total volume of some 30 ml., and the slurry poured into the chromatogram tube.

The tube was immediately rotated smartly between the hands to remove any air bubbles trapped between the particles of gel. The column was then allowed to pack down. During this stage it is important that the surface be kept covered with the developing solvent, for if allowed to dry, even for a short period, the column is liable to crack; should this occur the column is extruded, mixed with CB_1 and remade. The developing solvent must be added carefully without disturbing the surface of the column, as this leads to unevenness of the bands. When the column ceased to contract, 1 ml. of 0·03 N-*n*-valeric acid (Kahlbaum) in CB_1 was run on to the column and washed through with the same solvent. This served two purposes, (*a*) of testing the column for evenness of packing, and (*b*) of 'neutralizing' any local concentration of alkali, which occasionally appears as a result of uneven mixing of the alkaline indicator solution with the gel.

The behaviour of fatty acids on the chromatogram

Preliminary experiments with columns prepared as described above, and using CB_1 as the developing solvent, confirmed the observation of Lester Smith that the partition chromatogram would resolve a chloroform solution containing formic, acetic, propionic, *n*-butyric and *n*-valeric into its component acids. Formic acid remained fixed at the top of the chromatogram and the remainder passed down the column as discrete yellow bands, moving at different rates; *n*-valeric acid moved fastest and acetic acid the slowest. The leading edge of each band was sharp and well defined, whereas the rear edge tended to be diffuse and its termination not always easy to detect. Propionic, *n*-butyric and *n*-valeric acids were washed through the column with CB_1; but to remove acetic acid it was necessary to use CB_5 and this resulted in the leaching out of some of the indicator. Formic acid could only be washed out by raising the butanol concentration to 20% (v/v).

Martin & Synge (1941) demonstrated that, in the case of the partition chromatogram, R, the ratio between the rate of movement of a compound down the column and the rate of movement of the developing solvent in the tube above the chromatogram is related to the partition coefficient of the compound between water and the non-polar phase. The value R is thus characteristic of the compound. In the case of the volatile fatty acids it has been established that the partition coefficient varies with concentration (see Smith & White, 1929), and it is to be expected therefore that the R values of these compounds will vary in a similar fashion. In consequence, if the R value is to be used for the identification of the volatile fatty acids, the degree of variation must be determined over the range of concentrations likely to be met in practice.

Stock solutions of acetic, propionic, *n*-butyric and *n*-valeric acids (Kahlbaum) were made up in pure chloroform, and from these a suitable range of concentrations of each acid prepared. Samples of 1 ml. were used for the determination of the R value. The

sample was delivered on to the surface of a column with a pipette calibrated to deliver between two marks. When the sample had percolated into the gel the chromatogram was developed with CB_1; at intervals during the development the distances travelled both by the meniscus of the developing solvent and the leading edge of the band were measured, and the R values calculated. The values so obtained for different concentrations were plotted against concentration and the resulting curves, given in Fig. 1, show that the R values vary with

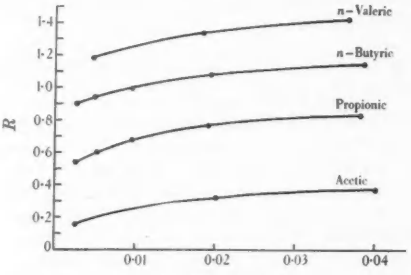

Fig. 1. The effect of concentration on the R value of fatty acids. (R = mm. moved by band/mm. moved by developing solvent in the tube above the chromatogram.)

concentration. To identify an unknown fatty acid therefore, it is necessary to determine both the R value and the concentration. In all probability, however, the method will not distinguish between *n*-butyric and *iso*-butyric acids on account of the similarity of their partition coefficients; nor, for the same reason, would it be possible to separate the four isomers of valeric acid.

The estimation of volatile fatty acids

Two preliminary steps are necessary before the chromatographic procedure can be applied to the analysis of volatile fatty acids in biological materials: (1) the volatile fatty acids must be separated from all other acidic substances, (2) the volatile fatty acids must be transferred quantitatively from aqueous solution to as small a volume of chloroform as possible. For stage (1) steam distillation is best, and of the numerous procedures available, the double distillation method of Friedemann (1938) has proved the most reliable. In the second distillation formic acid is oxidized so that the final distillate contains all the other volatile fatty acids. The exclusion of formic acid is desirable because it is difficult to remove from the column. Chemical methods are available for its estimation (Pickett, Ley & Zygmuntowicz, 1944; Dakin, Janney & Wakeman, 1913).

The fatty acids present in the distillate were quantitatively transferred to chloroform in the following manner. A drop of phenol red was added, the aqueous solution made alkaline with a slight excess of 0·1 N-NaOH, and evaporated to about 0·1–0·2 ml., the last stages being carried out in a long-necked 100 ml. round-bottomed flask heated on a boiling water-bath. Evaporation was facilitated by a blast of compressed air. When the fluid had been reduced to the desired volume, the flask was set aside to cool and drain. Finely powdered A.R. anhydrous $KHSO_4$ (3–4 g.) was added until a fairly dry, pink powder was obtained. The product was then loosened by tapping the flask and gently swirled to make certain that small amounts of liquid were not retained on the walls. The solid was now extracted with six 4 ml. portions of dry CB_5 and the combined extracts diluted to 25 ml. When pure chloroform or CB_1 was used the extraction was not quantitative.

The total volatile acids in this extract were determined as follows. Using a pipette calibrated to deliver between two marks, a suitable volume was transferred to a 6 × 1¼ in. boiling tube and *c.* 20 ml. distilled water added, followed by 1 drop of 0·1% phenol red as indicator. A brisk stream of CO_2-free air was bubbled through the aqueous phase for 3 min. (a sintered glass gas distributor was used to provide a stream of very fine bubbles). The acids were titrated with 0·01 N-NaOH from a 5 ml. burette graduated in 0·01 ml. with the current of CO_2-free air continued throughout the titration. Towards the end of the titration it was essential to shake the tube vigorously to complete the extraction of the acids from the solvent mixture; with these precautions a very sharp end-point was obtained. When large volumes of chloroform were used, emulsions sometimes formed which resulted in the addition of excess standard alkali, and it was found convenient to back titrate this excess with 0·01 N-acid. The efficiency of the extraction procedure can be judged by reference to column 1 of Table 1.

A suitable volume of the chloroform extract (see below) was pipetted on to a chromatogram, prepared as described in the previous section, and the chromatogram developed with CB_1. Each fraction except acetic acid was collected separately in a 6 × 1¼ in. boiling tube and titrated with 0·01 N-NaOH with the precautions outlined above. To ensure the complete recovery of each fraction it was necessary to collect the developing solvent from the time the leading edge of the first was about to leave the column until the leading edge of the succeeding band was about to leave. The determination of acetic acid by difference was necessary owing to the fact that CB_5, used to 'elute' acetic acid, leached considerable quantities of indicator, which obscured the end-point of the titration. This procedure is not

so satisfactory as a direct determination, but must continue to be employed until an indicator is found with the sensitivity of bromocresol green, but which, at the same time, is not leached by the developing solvent.

To assess the accuracy and precision of the method, 30 ml. of an aqueous solution containing known amounts of acetic, propionic and *n*-butyric acids were evaporated down and put through the extraction and chromatographic procedures outlined above. The results of fifteen such tests are given in Table 1. It will be seen that the recovery of each acid is adequate for most purposes.

Table 1. *Precision and accuracy of the determination of acetic, propionic and butyric acids in a mixture*

(Fifteen determinations were carried out on a standard mixture of the three acids and the results are expressed as the mean recovery and standard deviation in terms of 0·01 N-reagent. Volume of chloroform extract, 1 ml.)

	Total acid (ml.)	Butyric acid (ml.)	Propionic acid (ml.)	Acetic acid (ml.)
Theoretical	28·93	9·31	9·58	10·04
Observed	28·86±0·35	9·4±0·18	9·52±0·26	9·93±0·31

At the end of an estimation the chromatogram was freed from acetic acid by a thorough wash through with CB_5, followed by CB_1, and it was then ready for further use. In this way columns have been used as many as six times without any loss of performance, and in spite of the leaching of considerable quantities of indicator. A chromatogram, once made, can be preserved for a number of days by immersing the column in a tube of CB_1.

This chromatographic procedure has been applied over the past two years to the analysis of the volatile fatty acids in the rumen of the sheep, and has been found satisfactory.

Additional precautions. If good results are to be obtained with this technique, the following additional points should be borne in mind.

(1) If too large a volume of chloroform extract is taken for analysis on the partition chromatogram, the bands become broad and diffuse with a strong tendency to overlap. Table 2 records two experiments where 5 ml. samples were used; there was an apparent loss of some 30% of the *n*-butyric acid despite the fact that the recovery of total acid was good. This apparent loss was due to the fact that the butyric band was unnecessarily broadened and during the development the rear edge of the band was entirely engulfed by the leading edge of the propionic acid band. The overlapping was not due to the fact that the propionic acid was in excess of the *n*-butyric acid. The volume of chloroform extract taken for analysis should not exceed 3 ml. and should preferably be less.

Table 2. *Effect of volume of sample on the recovery of acetic, propionic and butyric acids*

(Volume of chloroform extract, 5 ml. Results in terms of 0·01 N-reagent.)

	Total acid (ml.)	Butyric acid (ml.)	Propionic acid (ml.)	Acetic acid (ml.)
Theoretical	86·5	9·5	47·2	29·9
Observed: (1)	86·3	7·22 (76%)	48·4 (102·5%)	30·7 (102·5%)
(2)	85·1	6·97 (73·5%)	48·9 (103·5%)	29·2 (97·5%)

(2) The 3 g. column should not be overloaded with acid, and the sample taken for analysis should not contain more than a total of 10 ml. of 0·01 N-acid.

(3) Each batch of gel should be thoroughly tested before being put to routine use. Recent batches of gel have necessitated the use of CB_5 throughout, and were observed to bind the indicator much more strongly than previous preparations; at the same time there was no evidence of loss of performance. The reason for this change in properties is as yet unknown.

SUMMARY

1. The silica-gel partition chromatogram, with bromocresol green as indicator, is found to resolve a chloroform solution of the acids formic, acetic, propionic, *n*-butyric and *n*-valeric.

2. The *R* values of the fatty acids vary with the concentration.

3. The procedure has been adapted for the identification and quantitative estimation of acetic, propionic and butyric acids in a mixture containing all three acids.

4. Formic acid cannot be estimated by this method and must be removed from the solution taken for analysis.

5. Elution of acetic acid is accompanied by the leaching out of the indicator in amounts sufficient to obscure the end-point in the final titration. Acetic acid therefore is determined by difference.

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The Action of Arsine on Blood

OBSERVATIONS ON THE NATURE OF THE FIXED ARSENIC

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A striking feature of poisoning by the inhalation of arsine is the haemolysis which occurs after a short latent period. It is known that arsine is 'fixed' in some non-volatile form by blood in the presence of oxygen and that this 'fixation' may be accompanied by changes in the colour and spectroscopic characteristics of the blood pigment, but little is known with certainty regarding the chemical changes involved in these phenomena and no satisfactory explanation of the mechanism of the haemolysis has been advanced.

That oxygen plays an essential part in the chemical changes was first indicated by Naunyn (1868), who showed that in its absence haemolysis did not occur in blood treated *in vitro* with arsine. This observation was repeatedly confirmed, e.g. by Meissner (1913), who recognized that arsine is fixed by blood in a non-volatile form in the presence of oxygen. He ascribed this fixation to oxidation to arsenious or arsenic acid, or to the formation of a complex compound, and discovered the important fact that haemoglobin is the only constituent of blood which will react vigorously with arsine. That the fixed arsenic is largely confined to the erythrocytes up to the time of commencement of haemolysis was made clear by the experiments of Thauer (1934), and this has been confirmed in experiments preliminary to the present work.

The chemical nature of the fixed arsenic formed in blood was considered as early as 1865 by Eulenberg, who examined the blood of an arsine-poisoned cat, and on the basis of precipitation experiments with magnesia mixture reported that arsenious acid but not arsenic acid was present. The use of such methods for the detection of small amounts of arsenious and arsenic acids in animal experiments was criticized by Joachimoglu (1916). Stricker (1882) also reported that arsine is oxidized in blood to arsenious acid, but we have been unable to consult the original work.

Labes (1928, 1937) suggested that in the presence of oxygen arsine becomes oxidized within the erythrocyte to colloidal elementary arsenic, which he supposed to be the active haemolytic agent. He observed that colloidal solutions of elementary arsenic haemolyze erythrocyte suspensions, but presented no direct evidence that intracellular colloidal arsenic is formed when blood is treated with arsine.

Extensive studies of arsine fixation by haemoglobin and of the nature of the haemolytic agent have been made by Heubner and his co-workers, who allowed whole blood, erythrocyte suspensions, or haemoglobin solutions to react with arsine in the presence of oxygen. From the arsenic content of the reaction products and from the amount of oxygen utilized surmises as to the probable nature of the fixed arsenic were made. Heubner & Wolff (1936) considered that arsenic dihydride ($AsH_2)_2$, was first formed and that this compound was the haemolytic agent. Their figures further indicated that the final product of oxidation of arsine in the presence of haemoglobin was arsenious acid. Wolff (1936) agreed that this was the final product, but suggested that the intermediate compound was hydroxyarsine (AsH_2-OH). Gebert (1937) suggested that arsenic dihydride was formed as an intermediate in the oxidation of arsine to elementary arsenic, the latter being eventually further oxidized to 'arsenik'.

These conclusions of Labes and of the Heubner school seem to be coloured by the assumption that an oxidation product of arsine must necessarily be the direct haemolytic agent.

In the present work an attempt has been made to gain a further insight into the chemical mechanisms involved in the arsine-blood reaction, by a direct examination of the properties and behaviour of the fixed arsenic formed in the blood of arsine-poisoned rabbits, and in normal rabbit blood and haemoglobin solutions treated *in vitro* with arsine.

EXPERIMENTAL

Methods

Arsenic determination. Total arsenic was determined by the micro-method of Levvy (1943). Samples contained 10–30 μg. As. Analyses were carried out in duplicate or triplicate. The figures shown in the tables were calculated from the means of the values obtained.

Technique of animal experiments. Rabbits were exposed to the desired concentration of arsine in air in a specially designed chamber which permitted the instantaneous exposure of the animal to the full concentration of gas and its instantaneous withdrawal to an uncontaminated atmosphere after the exposure period. Each animal was immediately stunned by a blow on the head and rapidly bled, by slitting its throat, into a large beaker surrounded by ice. In some cases the blood was defibrinated with a wire brush; in other cases clotting was prevented by injecting the animals with heparin before stunning and bleeding them into beakers containing more heparin. After measuring the volume of blood, samples (usually 1 ml.) were withdrawn for determinations of total As and of percentage haemolysis; the remainder was then laked by the addition of 2 vol. of distilled water. In no instance was any haemolysis detectable in the freshly drawn blood up to the time of laking. Stromata were removed as completely as possible from the laked blood (henceforward referred to as 'the haemolysate') by centrifuging.

Technique of in vitro *experiments with blood.* Blood from a heparinized rabbit was placed in a 500 ml. round-bottomed flask fitted with a rubber stopper through which was passed a short glass tube. A 2 in. length of thin-walled rubber tubing was fitted over the external end of the glass tube, and the other end of the rubber tube closed by a glass rod. The vessel was submerged in a constant-temperature bath at 37° and the arsine injected by hypodermic syringe through the rubber tubing into the flask. After shaking at 37° for 5–10 min. any unreacted arsine was removed by thorough evacuation. After removal of samples for analysis, haemolysates were prepared as described above.

Dialysis of haemolysates obtained from arsine-poisoned blood

In an attempt to obtain information as to whether the fixed As in the erythrocyte is merely a simple oxidation product of arsine, or whether it is in the form of a complex with one of the cell constituents, an extensive series of quantitative experiments on the dialysis of haemolysates prepared from arsine-poisoned rabbits and from normal rabbit blood treated *in vitro* with arsine was carried out. The only previous reference to any similar work which can be traced is the following statement by Heubner & Wolff (1936): 'Bei der Dialysen einer mit Arsenwasserstoff behandelten Blutprobe geht ins Dialysat nicht viel weniger Arsen über als aus einer gleichen mit Arsenik versetzen Probe.' No experimental details of this work were reported, and no further reference to it has been made in later papers from Heubner's laboratory.

After removal of samples from each haemolysate for the determination of total As, additional samples (usually 3 ml.) were placed in small dialysis sacs made from 'Visking' synthetic sausage casing (*c.* $\frac{3}{4}$ in. diam.) and dialyzed for 48 hr. in the refrigerator against four changes of 100 ml. water. The As remaining in each sac and its contents was then determined, blank determinations being simultaneously carried out on empty sacs.

Preliminary experiments showed that under these conditions of dialysis, the dialyzable As is completely removed from haemolysates of arsine-poisoned blood in less than 48 hr. The results from fourteen experiments on arsine-poisoned rabbits (Table 1) and from eight experiments with the blood

Table 1. *Non-dialyzable arsenic in haemolysates from arsine-poisoned rabbits*

No. of exp.	AsH_3 conc. (mg./l.)	Time of exposure (min.)	Total As in blood (μg./ml.)	Total As in haemolysate (μg./3 ml.)	As in haemolysate after dialysis (μg./3 ml.)	Non-dialyzable As in haemolysate (%)
1	0·46	20	13·7	12·8	12·5	98
2	0·46	20	17·2	17·0	17·7	104
3	0·53	20	—	15·4	9·7	63
4	0·46	20	10·6	10·8	10·7	99
5	0·47	20	9·2	8·9	5·6	63
6	0·51	20	15·7	16·2	13·8	85
7	0·47	20	22·7	16·8	14·5	86
8	0·48	20	22·4	20·5	17·5	85
9	0·50	20	31·9	24·1	13·0	54
10	0·46	20	14·9	14·7	11·5	78
11	0·95	20	38·4	33·7	34·1	101
12	0·91	20	40·4	37·6	31·2	83
13	0·75	20	—	21·5	20·9	97
14	0·94	5	—	9·8	6·4	65

Table 2. *Non-dialyzable arsenic in haemolysates from blood of normal rabbits treated* in vitro *with arsine*

No. of exp.	Total As in blood (μg./ml.)	Total As in haemolysate (μg./3 ml.)	As in haemolysate after dialysis (μg./3 ml.)	Non-dialyzable As in haemolysate (%)
15	10·5	9·8	5·1	52
16	9·6	5·8	4·7	56
17	37·6	36·4	21·8	60
18	48·3	51·4	27·9	54
19	54·1	45·8	21·4	47
20	47·9	41·4	22·2	54
21	—	54·3	31·0	57
22	—	53·3	26·2	49

of normal rabbits treated *in vitro* with arsine (Table 2) clearly show that the fixed As in arsine-poisoned blood can be separated into two distinct fractions. It is noteworthy that in the experiments on the haemolysates from poisoned rabbits the non-dialyzable As varied from 54 to 100 % of the total As, while in the experiments on the haemolysates from *in vitro* poisoned rabbit blood, the non-dialyzable As varied only between 47 and 60 % of the total. It is very tentatively suggested that the less constant and generally higher values found for the

non-dialyzable As in the *in vivo* poisoning experiments may have been due to diffusion of dialyzable As out of the erythrocytes and subsequent loss to the tissues or through the kidneys during the period of exposure in the gas chamber.

The nature of the dialyzable fixed arsenic in arsine-poisoned blood

The method of quantitative separation of arsenate and arsenite developed by Crawford & Storey (1944) has been applied to an examination of the dialyzable fixed As present in the haemolysate obtained from one arsine-poisoned rabbit and in three haemolysates from normal rabbit blood treated *in vitro* with arsine. This method, which depends upon the fact that arsenious acid, but not arsenic acid, forms a CCl_4-soluble compound when treated with a CCl_4 solution of sodium ethyl xanthate, was found to give good recoveries of arsenite and arsenate when these substances were added to normal rabbit blood.

The main bulk of each haemolysate (50–100 ml.), after removal of samples for total As and non-dialyzable As, was dialyzed in a large 'Visking' sausage casing for 24 hr. in the refrigerator against two lots of approximately 100 ml. of water. The two dialysates were then combined and made up to 250 ml. Samples were removed for total As, and 'arsenate' and 'arsenite' determined in the remainder by the xanthate method. As a precaution, the acidified dialysate was extracted three times with CCl_4 before extraction with the reagent.

The figures for 'arsenite' and 'arsenate' in the dialysates of the four experiments (Table 3) were obtained from the values actually determined by applying a correction factor derived from the total dialyzable As in the haemolysate and the total As in the combined dialysates. These results show that

Table 3. *'Arsenite' and 'arsenate' in the dialyzable fixed arsenic in arsine-poisoned blood*

No. of exp.	Type of exp.	Total As in haemolysate (μg./100 ml.)	Total dialyzable As in dialysate (μg./100 ml. haemolysate)	'Arsenite' and 'arsenate' as As in dialysate (μg./100 ml. haemolysate)	
				'Arsenite'	'Arsenate'
12	Poisoned rabbit	1250	210	155	40
19	Rabbit's blood	1530	817	725	0
20	poisoned	1380	640	534	0
21	*in vitro*	1810	780	780	29

in both types of experiment a high proportion of the dialyzable fraction of the fixed As behaves towards the xanthate reagent as arsenite.

Evidence for the existence of two forms of non-dialyzable arsenic in arsine-poisoned blood

With the object of finding a method of rendering the non-dialyzable As fraction dialyzable, so that its behaviour towards the xanthate reagent could be studied, many preliminary experiments on the stability of this fraction were made.

The stability of the non-dialyzable As in the haemolysate from the blood of a poisoned rabbit to dialysis in the refrigerator against solutions of different pH was first studied. Over the range pH 2–11 the non-dialyzable As appeared to be stable for at least 6 days. On the other hand, after 6 days' dialysis at pH 1 (0·1 N-HCl), 77 % of the normally non-dialyzable As had dialyzed out, while after 6 days at pH 13 (0·1 N-NaOH), 53 % dialyzed. The addition of a little dilute $K_3Fe(CN)_6$ solution caused a large proportion, but not all, to become dialyzable. A smaller proportion was rendered dialyzable by heating the haemolysate to boiling for a few minutes, and about the same proportion by adding pyridine to give a concentration of about 40 %.

It was striking that in none of these preliminary, somewhat qualitative, experiments was all the non-dialyzable As rendered dialyzable. This suggested the possibility that there might be present in the haemolysates from arsine-poisoned blood two forms of non-dialyzable As differing from one another in stability. This possibility was tested by more quantitative experiments on the conversion of non-dialyzable to dialyzable As by the ferricyanide method.

(1) The haemolysate from the blood of an arsine-poisoned rabbit was dialyzed for 3 days against distilled water, the water being changed three times daily. Six 3 ml. samples of the dialyzed haemolysate were dialyzed in 'Visking' dialysis sacs in the refrigerator against 5 % $K_3Fe(CN)_6$. Two sacs were removed each day for the determination of undialyzed As. Four 3 ml. samples of the haemolysate were dialyzed against distilled water as controls, the water being changed three times daily. Two samples were removed after the first day, and the remaining two after the third day for determination of As. Blank determinations on dialysis sacs containing 3 ml. of distilled water and dialyzed against 5 % $K_3Fe(CN)_6$ were carried out.

(2) A second experiment was carried out in an identical manner with the dialyzed haemolysate from normal rabbit blood which had been poisoned *in vitro* with arsine.

(3) Since in Exps. 1 and 2, owing to a miscalculation in the size of the samples taken for dialysis against ferricyanide, the amounts of As actually estimated were in some cases considerably less than 10 μg., a third similar experiment was carried out on a dialyzed haemolysate from the blood of a poisoned rabbit, but the samples taken for dialysis against ferricyanide were of such a size as to ensure that at least 20 μg. As would be estimated.

The results of these three experiments, which are shown in Table 4, make the conclusion almost inescapable that about 70–80 % of the non-dialyzable As differs from the remainder in being readily converted to a dialyzable form by treatment with ferricyanide.

Table 4. *Dialysis against 5 % potassium ferricyanide of 'water-dialyzed' haemolysates from arsine-poisoned blood*

Type of exp.	Non-dialyzable As in haemolysate (μg./3 ml.)	Undialyzed As remaining after dialysis against ferricyanide for (μg./3 ml. haemolysate) 1 day	2 days	3 days	Controls. As after dialysis against water for (μg./2 ml. haemolysate) 1 day	3 days
In vivo	20·9	5·6	3·3	3·0	21·0	18·1
In vitro	26·2	11·4	6·0	8·4	29·6	32·0
In vivo	29·1	10·6	7·5	6·7	29·5	24·7

The nature of the labile non-dialyzable arsenic in arsine-poisoned blood

Certain of the methods described in the preceding section for the conversion of part of the non-dialyzable As to the dialyzable form were unsuitable as preliminaries to examination by the xanthate reagent. Kolthoff (1919) showed that in alkaline solution arsenite becomes rapidly oxidized to arsenate. Control experiments conducted in the course of the present work showed in fact that in the presence of blood arsenite is rapidly oxidized to arsenate at pH 13. Conversion at pH 1 was also considered to be unsuitable, since the proportion of non-dialyzable As rendered dialyzable at this reaction was found to vary considerably from experiment to experiment. Conversion in 40 % pyridine was also rejected, since it was considered that the large amount of pyridine present in the dialysate might interfere with the fractionation. However, preliminary experiments showed that both boiling and treatment with ferricyanide would be satisfactory as preliminaries to the examination of the labile non-dialyzable As by the xanthate reagent. Sodium arsenite or arsenate was added to rabbit blood, and after laking with water the haemolysates were boiled or treated with ferricyanide. Examination of the dialysates from these treated haemolysates showed that no conversion of arsenite to arsenate, or vice versa, had occurred.

A haemolysate obtained from the blood of an arsine-poisoned rabbit was freed as completely as possible from dialyzable As by dialysis in the refrigerator for 4 days against seven changes of distilled water. 50 ml. of this dialyzed haemolysate were placed in a large 'Visking' dialysis sac which was immersed in a beaker containing about 100 ml. of boiling water for 10 min., and then the beaker and dialysis sac were transferred to the refrigerator. After 24 hr. the water in the beaker was changed and dialysis continued for a further 24 hr. The two dialysates were combined, and after making up to a volume of 250 ml., determinations of 'arsenite' and 'arsenate' by the xanthate method, and of total As were carried out.

To a further 45 ml. of the original dialyzed haemolysate were added 20 ml. of 5 % $K_3Fe(CN)_6$. After standing at room temperature for 20 min. the mixture was transferred to a dialysis sac and dialyzed for 48 hr. in the refrigerator against two 100 ml. lots of distilled water. The combined dialysates were made up to 250 ml. and determinations of 'arsenite', 'arsenate', and total As carried out.

A second similar experiment was carried out on a haemolysate prepared from normal rabbit blood poisoned *in vitro* with arsine.

The results (Table 5) show quite clearly that a high proportion of the dialyzable As obtained from the non-dialyzable form by boiling and by ferricyanide treatment behaves towards the xanthate reagent as arsenite.

Table 5. *'Arsenite' and 'arsenate' in dialysates obtained from water-dialyzed haemolysates of arsine-poisoned blood after boiling and after treatment with potassium ferricyanide*

Type of exp.	Boiling: Total As in dialysate (μg.)	Boiling: As in dialysate as 'arsenite' (μg.)	Boiling: As in dialysate as 'arsenate' (μg.)	Ferricyanide: Total As in dialysate (μg.)	Ferricyanide: As in dialysate as 'arsenite' (μg.)	Ferricyanide: As in dialysate as 'arsenate' (μg.)
In vivo	187	185	8	353	307	10
In vitro	190	168	17	179	153	18

Experiments with crystalline horse haemoglobin

Certain of the findings described in the preceding sections have been confirmed in experiments on solutions of crystalline horse haemoglobin treated with high concentrations of arsine.

Preparation of haemoglobin solutions and technique of treatment with arsine. Erythrocytes from oxalated fresh horse blood were washed eight times with 0·9 % NaCl and then laked by making up to the original volume of the blood with distilled water. This haemolysate was frozen in large centrifuge cups which were then spun until thawing was just complete. After pouring off the supernatant liquid, the sludge, consisting of haemoglobin crystals and stromata, was shaken with 0·9 % NaCl and the stromata were removed by centrifuging. The concentration of haemoglobin in the solution was determined by the alkaline-haematin method.

A known amount of arsine was introduced into a round-bottomed 3 l. flask by displacement of a measured volume of water with a hydrogen-arsine mixture of known arsine content. A measured volume of haemoglobin solution was then rapidly run into the flask from a burette under slight pressure. The flask was shaken continuously for a period of 20–35 min., and any unreacted arsine was subsequently removed by thorough evacuation at the water-pump.

Non-dialyzable fixed arsenic in arsine-treated haemoglobin solutions and the behaviour of the dialyzable arsenic fraction towards the xanthate reagent. Four experiments were carried out as described above in which haemoglobin solutions were gassed for varying periods with varying concentrations of arsine. From each reaction mixture measured

samples were removed for the determination of total fixed As and of non-dialyzable fixed As. The results (Table 6) indicate that as was the case with poisoned blood, a proportion of the total fixed As is in a non-dialyzable form. This proportion was somewhat lower than that found for blood.

Table 6. *Non-dialyzable arsenic in solutions of horse haemoglobin treated with arsine*

Reaction period (min.)	Haemoglobin content of solution (g./100 ml.)	AsH_3 added (mg./100 ml.)	Total As in reaction mixture (mg./100 ml.)	Non-dialyzable As in reaction mixture (mg./100 ml.)	Non-dialyzable As as % of total As
20	1·28	11·3	9·59	3·95	41
20	1·28	11·3	9·98	3·98	40
35	2·50	178·0	32·3	10·7	33
35	2·50	178·0	51·6	18·3	35

'Arsenite' and 'arsenate' in the dialyzable fractions in each of the four experiments were determined in the following manner: One volume of the reaction mixture (50–150 ml.) was dialyzed for 24 hr. in the refrigerator in a 'Visking' dialysis sac against approximately 2 vol. of water. After adjustment of the dialysate to a suitable volume, measured samples were removed for the determination of total As and of 'arsenite' and 'arsenate' by the xanthate method. In the four experiments 97·0, 97·2, 97·5 and 96·4 % respectively of the total As in the dialysate was found to behave towards the xanthate reagent as arsenite.

SUMMARY

1. 50–100 % of the fixed arsenic in the blood of arsine-poisoned rabbits is in a non-dialyzable form.

2. 47–60 % of the arsenic fixed in normal rabbit blood treated *in vitro* with small concentrations of arsine in the presence of excess air is in a non-dialyzable form.

3. Of the fixed arsenic present in solutions of crystalline horse haemoglobin after treatment with high concentrations of arsine in the presence of excess air, from 33–41 % was found to be non-dialyzable.

4. The dialyzable arsenic present in the blood of arsine-poisoned rabbits, in normal rabbit blood and in solutions of crystalline horse haemoglobin treated *in vitro* with arsine, was almost completely in a form which behaved as arsenite towards the xanthate reagent.

5. The non-dialyzable arsenic present in rabbit blood poisoned *in vitro* and *in vivo* with arsine is stable between pH 2 and 11, but is partially converted to a dialyzable form at pH 1 and 13. Partial conversion to a dialyzable form is also effected by treatment with pyridine, ferricyanide, or by boiling.

6. Quantitative experiments on the effect of ferricyanide on the non-dialyzable fraction showed that only 70–80 % of this fraction could be rendered dialyzable. It is concluded that there are present two forms of non-dialyzable arsenic.

7. Most of the non-dialyzable arsenic rendered dialyzable by ferricyanide or boiling behaves as arsenite towards the xanthate reagent. The 'labile' non-dialyzable arsenic fraction is therefore probably in the trivalent form.

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The Free Amino Group of Gramicidin S

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Synge (1945) has recently shown that gramicidin S is a polypeptide composed of equimolecular proportions of the five amino-acids, *l*-valine, *l*-proline, *l*-leucine, *l*-ornithine and *d*-phenylalanine, and that each stoichiometric unit contains one free amino group. Belozersky & Paschina (1944*a*, *b*, 1945), on the other hand, maintain that both amino and carboxyl groups are present and that the δ-amino group of the ornithine residue is not free. Their evidence for this latter point, nevertheless, is not very convincing and the problem was clearly one which could be readily settled by applying the new dinitrofluorobenzene (DNFB) method (Sanger, 1945). Accordingly at Dr Synge's suggestion, the present author has made appropriate experiments, which show that the free amino group is undoubtedly the δ-amino group of the ornithine residue. The finding provides additional support for Synge's contention that gramicidin S is a *cyclo*peptide composed of the five amino-acids linked through their α-amino and carboxyl groups. It is interesting to note that similar results for tyrocidin have recently been obtained by Christensen (1945) using a different technique.

EXPERIMENTAL

Preparation of compounds

δ-2:4-*dinitrophenyl*-l-*ornithine* was prepared by treatment of the copper complex of *l*-ornithine with DNFB as follows. 0·1 g. *l*-ornithine monohydrochloride, dissolved in 5 ml. hot water, was treated with excess $CuCO_3$. After filtration the solution was evaporated to about 2 ml., and 0·3 g. $NaHCO_3$ was added followed by a solution of 0·2 ml. DNFB in 4 ml. ethanol. The mixture was shaken for 2 hr. at room temperature, which brought about the separation of the copper complex of δ-DNP-ornithine as a green powder. This was filtered off, dissolved in dilute HCl, treated with H_2S and filtered with charcoal. The solution was then taken to a small volume *in vacuo*, which brought about the separation of the *hydrochloride of* δ-2:4-*dinitrophenyl*-l-*ornithine* as crystals containing one molecule of water of crystallization. It was recrystallized from N-HCl; yield 0·09 g., m.p. 223° (decomp.). (Found: C, 38·0; H, 5·0; N, 10·0; Cl, 16·4%. $C_{11}H_{14}O_6N_4.HCl.H_2O$ requires C, 37·5; H, 4·9; N, 10·1; Cl, 15·9%.) It lost 5·0% moisture at 100° over P_2O_5 (calc. 5·1%).

When an acid solution of the above hydrochloride was neutralized with pyridine in an attempt to prepare the free amino-acid, there was a slow separation of an oil which would not crystallize, although with the corresponding *dl*-compound the free amino-acid could readily be prepared crystalline in this way. A similar difficulty was encountered in the preparation of ε-DNP-*l*-lysine; ε-DNP-*dl*-lysine has not yet been studied.

α-2:4-*dinitrophenyl*-dl-*ornithine* was prepared by treatment of δ-benzoyl-*dl*-ornithine with DNFB, followed by hydrolysis of the benzoyl group. 0·3 g. *dl*-ornithine monohydrochloride was converted to the copper complex, and the cooled solution benzoylated in the usual manner with 0·32 ml. benzoyl chloride and 6 ml. N-NaOH. The insoluble benzoyl compound was filtered off, suspended in 2 ml. water and treated with H_2S. This solution was brought to the boil and filtered hot. On evaporation of the filtrate to about 5 ml., 0·14 g. δ-benzoyl-*dl*-ornithine crystallized. This was suspended in 2 ml. water containing 0·3 g. $NaHCO_3$ and shaken for 2 hr. with a solution of 0·2 ml. DNFB in 4 ml. ethanol. After removal of the ethanol *in vacuo*, the solution was extracted with ether to remove excess DNFB and acidified. The α-DNP-δ-benzoyl-*dl*-ornithine immediately separated as an amorphous solid; yield 0·2 g. For the removal of the benzoyl group it was heated for 4 days at 105° in an evacuated sealed tube with a mixture of 2 ml. 10N-HCl and 2 ml. acetic acid. After cooling, the solution was taken to dryness and the residue dissolved in water. Unhydrolyzed material, of which there was still a considerable amount, was then extracted with ethyl acetate, and on neutralization of the aqueous solution with pyridine 0·05 g. α-2:4-*dinitrophenyl*-dl-*ornithine* crystallized out; m.p. 227° (decomp.). (Found: C, 44·1; H, 4·9%. $C_{11}H_{14}O_6N_4$ requires C, 44·3; H, 4·7%.)

2:4-*dinitrophenyl-gramicidin S*. 0·1 g. Gramicidin S (specimen 1 of Synge, 1945) was dissolved in 4 ml. ethanol. 0·4 g. $NaHCO_3$, 1 ml. water and 0·2 ml. DNFB were added and the mixture shaken for 3 hr. The DNP derivative of gramicidin S crystallized as the reaction proceeded. It was recrystallized from ethanol water; yield 0·1 g.

Identification of the free amino group of gramicidin S

5 mg. DNP-gramicidin S were heated for 54 hr. at 110° in a sealed evacuated tube with a mixture of 1 ml. 10N-HCl and 1 ml. acetic acid. The hydrolysate was taken to dryness, dissolved in water and extracted with ether. Since the whole of the colour remained in the aqueous solution, the presence in this solution of a mono-DNP derivative of ornithine was indicated. Any other DNP derivative of the amino-acids present in gramicidin S would have been extracted by the ether (Sanger, 1945). It remained only to identify the ornithine derivative present.

It was found that α-DNP-ornithine and δ-DNP-ornithine moved on a 66% methyl ethyl ketone (MEK)-ether column at $R = 0{\cdot}07$ and 0·12 respectively (Gordon, Martin & Synge, 1943), and thus could be separated from one another. The material

from DNP-gramicidin S moved at $R = 0{\cdot}12$, and when mixed with α-DNP-ornithine, two bands were formed having $R = 0{\cdot}12$ and 0·07 respectively. This shows that δ-DNP-ornithine and no other DNP derivative was present, and that the only free amino group present in gramicidin S is the δ-amino group of the ornithine residue.

In a further quantitative experiment, the δ-DNP-ornithine in a hydrolysate of DNP-gramicidin S was estimated colorimetrically, using synthetic δ-DNP-*l*-ornithine hydrochloride as a standard. A parallel experiment showed that the conditions of hydrolysis lead to no appreciable breakdown, so it was not necessary to make any correction for this. The yield of δ-DNP-ornithine in duplicate experiments was 35·0 and 35·8 % of the DNP-gramicidin S taken. If gramicidin S is a *cyclo*pentapeptide, the theoretical yield would be 40·5 %.

Isolation of δ-DNP-ornithine from DNP-gramicidin S

100 mg. DNP-gramicidin S were hydrolyzed as described above, taken to dryness and dissolved in N-HCl. After extraction with ether and filtration, the solution was allowed to evaporate at first *in vacuo* and then slowly in a desiccator, whereupon the δ-DNP-ornithine hydrochloride crystallized out, and was recrystallized four times from 5N-HCl; yield 5·7 mg. The various mother liquors were then combined and purified on a 66 % MEK-ether column. Only material from the centre dark portion of the band was collected. This solution was taken to dryness and the residue dissolved in a minimum volume of hot 5N-HCl, a trace of charcoal added and the solution filtered hot. On cooling, 6·2 mg. of the hydrochloride crystallized out, and a further 2·9 mg. was obtained from the mother liquors, making a total yield of 14·8 mg. (31 % theoretical).

*Crystallographic examination of δ-DNP-*l*-ornithine hydrochloride*

This material and the synthetic δ-DNP-*l*-ornithine hydrochloride were investigated crystallographically by Mr G. M. J. Schmidt of the Department of Chemical Crystallography, Oxford. He found that 'the synthetic preparation consisted of both plates and long thin needles, whereas the product from natural sources was mainly in the form of needles. However, both X-ray and optical examination showed the two preparations to be identical, the difference in crystal habit being presumably due to differences in the composition of the solutions from which the crystals were grown.

'The crystals are yellow orthorhombic needles or plates elongated along [010], the former showing {001} and {101}, whereas the latter show {100} and {001}. The orientation of the three principal refractive indices is $\alpha//c$, $\beta//b$, $\gamma//a$. The unit cell dimensions are $a = 10{\cdot}08$ A., $b = 4{\cdot}88$ A., $c = 32{\cdot}9$ A. Space group $P2_12_12_1$. ρ calculated 1·4 for $n = 4$.'

CO_2 *liberated by interaction between ninhydrin and DNP derivatives of amino-acids*

In Table 1 are shown the values for the CO_2 liberated by ninhydrin (Van Slyke, Dillon, MacFadyn & Hamilton, 1941) from the above material and from various synthetic derivatives. These analyses were carried out by Dr R. L. M. Synge.

Table 1. CO_2 *liberated by ninhydrin from various DNP derivatives (Synge)*

Compound	CO_2 found (% of 1 m-equiv.)
DNP-glycine	2
α-DNP-*dl*-ornithine	4
δ-DNP-*l*-ornithine.HCl.H_2O (synthetic)	97
Product from gramicidin S	92

The reaction was effected with 50 mg. ninhydrin at pH 2·5 and volume 1·3 ml. They confirm that the derivative from gramicidin S is δ-DNP-ornithine and not α-DNP-ornithine.

SUMMARY

The free amino group of gramicidin S is the δ-amino group of the ornithine residue, and there are no other free amino groups. Gramicidin S is thus a *cyclo*peptide.

I wish to thank Mr G. M. J. Schmidt for carrying out the crystallographic examinations, and Dr R. L. M. Synge for his help and advice with the work.

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A Note on the Action of Tannin upon Tumour Glycolysis

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Considerations dealing with the relation between cell proliferation and carbohydrate metabolism (particularly that of glucose), as well as with certain effects of variation in the water content of growing tissue, have suggested that the intensity of carbohydrate breakdown in proliferating cells is largely dependent upon the degree of hydration of corresponding enzyme proteins, in such a manner that, within limits, an increase in hydration stimulates and a decrease inhibits enzymatic activity (Lasnitzki, 1928, 1945). This assumption is supported by the results of investigations on the influence of hydrating and dehydrating ions upon glycolysis and related processes (see Lasnitzki, 1945).

Some further evidence in favour of this assumption, obtained in a different way, is recorded in this note. It was felt desirable to carry out similar investigations with substances whose mode of action differed from that of the electrolytes, although under such conditions research might be confined mainly to the study of the effects of dehydration. The most suitable agents appeared to be represented by the group of vegetable tannins, having regard to the available evidence of their dehydrating power, in low concentrations, upon proteins and other hydrophilic colloids (Kruyt, 1922; Bungenberg de Jong, 1923, 1924), irrespective of the nature of the underlying chemical changes. Experiments on the action of a tannin upon the anaerobic glycolysis of tumour tissue were thus undertaken, and ordinary tannic acid was chosen as the agent to be tested. A transplanted rat tumour, Jensen sarcoma, served as material.

EXPERIMENTAL

Methods

The preparation of the tumour tissue and measurement of glycolysis were carried out according to Warburg (1930). The tissue slices, obtained from the peripheral (non-necrotic) zone of the tumour, were placed in Ringer's solution which contained, apart from NaCl and KCl in the usual concentrations, 0·025 M-bicarbonate and 0·2 % glucose, but was free of Ca in order to avoid formation of sparingly soluble calcium tannate. A similar Ringer's solution (1·0 ml.), but of double concentration, was then pipetted into each of two metabolism vessels. To the fluid in vessel 1 was added 1·0 ml. of distilled water to make up 2·0 ml. of control Ringer's solution; while the fluid in vessel 2 was similarly mixed with 1·0 ml. of a dilute, freshly made solution of tannic acid in distilled water, a commercial tannic acid preparation of high purity being employed. Within a few seconds the tissue slices were immersed in the media thus prepared and the latter were immediately saturated with about 5 % CO_2 in N_2 (pH ~7·4). This procedure prevented the partial oxidation of tannic acid in the Ringer's solution during the preparatory period of the experiment. The measurements were performed at 37·5°.

Results

The results of four experiments, in which the effect of 0·1 % tannic acid was tested, are given in Table 1. It will be seen that the rate of glycolysis in the tannin-containing medium was clearly decreased as compared with that in the control medium. In Exps. 3 and 4 the percentage inhibition was little different during the first and second 30 min. of observation, but in Exp. 2 the inhibition during the latter interval was about twice as great as during the former. The average inhibition of glycolysis was approximately 40 %. Exps. 3 and 4, which were made on slices from the same tumour, showed a fair agreement in corresponding values; the average course of lactic acid formation in these

Table 1. *Effect of* 0·1 % *tannic acid on the glycolysis of Jensen rat sarcoma*

	Glycolysis, $Q_G^{N_2}$					
	Ringer's solution (control)		Ringer's solution +0·1 % tannic acid		Inhibition by tannin (%)	
Exp. no.	First 30 min.	Second 30 min.	First 30 min.	Second 30 min.	First 30 min.	Second 30 min.
1	26·3	—	16·2	—	38	—
2	30·8	28·0	22·8	13·6	26	51
3	26·8	22·4	17·8	12·9	34	42
4	27·2	22·8	17·1	13·0	37	43

Under conditions similar to the above, tannin-containing Ringer's solution, without tissue, showed a minute but continuous increase in manometric pressure, probably due to CO_2 liberation indicating a slight hydrolysis of tannic acid. In calculating the rates of glycolysis, this extra CO_2, amounting to 2–3 μl./ml. of solution/hr., was allowed for in nos. 2–4, but not in no. 1. In nos. 3 and 4 the bicarbonate content of the Ringer's solution to which the tannic acid had been added was increased to 0·028 M, in order to compensate for the loss of bicarbonate caused by that addition (amounting to about 10 %).

two experiments, for the control and the tannin-containing medium, is illustrated in Fig. 1. In a further experiment, in which the tannic acid content of the medium was raised to 0·2%, the inhibition of glycolysis was about 60%. It is evident, therefore, that tannic acid, in low concentration, has a marked inhibitory effect upon the glycolytic activity of tumour tissue. It is to be noted that the rate of glycolysis in the control medium (see

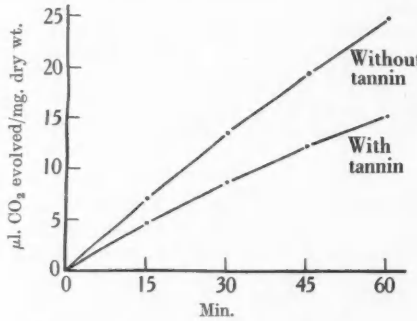

Fig. 1. Glycolysis of Jensen rat sarcoma in Ringer's solution, with and without addition of tannin (0·1% tannic acid).

specially Exps. 1, 3 and 4) was here somewhat lower than that normally observed, owing to the use of calcium-free Ringer's solution, the lack of calcium causing an outward diffusion of potassium from the tumour cell, which in turn decreases glycolysis to some extent (Lasnitzki, 1938).

At the end of the experiments the tissue slices removed from the tannin-containing medium were somewhat turbid in appearance as compared with the fairly transparent slices removed from the control medium.

DISCUSSION

The process of 'protein tanning' very likely consists in a chemical combination of tannin with the protein concerned, the nature of this combination being not yet fully understood. The protein molecule thus changed shows physical properties essentially different from those of the normal protein molecule, and the most important of these alterations appears to be its partial or total conversion into a hydrophobic colloid. It is an obvious assumption that the series of enzyme proteins involved in the anaerobic breakdown of glucose by the tumour cell will, under the influence of tannic acid, be altered in a similar manner, and there is a probability that not the chemical change *per se*, but the associated dehydration of the enzyme protein molecule is the factor responsible for the inhibitory effect of tannic acid upon that process. This view agrees with the supposed connexion between enzymatic carbohydrate breakdown in proliferating cells and the degree of hydration of corresponding enzyme proteins, as is substantiated by the investigations already mentioned on the action of ions.

The experiments described, which were carried out some time ago, have a bearing on more recent investigations by Powell (1944), who found that the growth of a transmissible mouse carcinoma could be strongly inhibited by tannic acid, among other substances, when a low concentration (especially as sodium salt) was administered continuously by intraperitoneal injection. These findings are in agreement with the result that tannic acid, in low concentration, is likewise able to cause a definite inhibition of tumour glycolysis.

SUMMARY

The anaerobic glycolysis of tumour tissue (Jensen rat sarcoma) can be markedly inhibited by tannic acid in low concentration.

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An Electrophoretic Examination of Cobra Venoms

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(*Received* 31 *December* 1945)

The complex chemical nature of Indian cobra (*Naja naja*) venom was indicated by Ganguly & Malkana (1936) and Ganguly (1937), who reported its percentage composition as follows: total protein 87·56, globulin 20·31, albumin 39·69, primary proteose 11·31, secondary proteose 16·81. In addition to proteins, the venom was found to contain cholesterol, phospholipins, inorganic substances and colouring matter. The toxic principle was found to be associated with the secondary proteose. Ganguly (1936) also reported the presence of proteolytic, lecithin-splitting and rennin-like enzymes in the venom.

The work recorded in this article is the result of an investigation of several cobra venoms by the method of electrophoresis.

MATERIALS AND TECHNIQUE

Venoms of the following snakes were investigated: (1) *Naja nigricollis* (M'fesi or spitting cobra), (2) *N. haja* (Egyptian cobra), (3) *N. naja* (Indian cobra), (4) *N. flava* (Cape cobra), (5) *Sepedon haemachates* (black ringhals).

Desiccated venoms were obtained from the South African Institute for Medical Research, Johannesburg, and several samples of venom in fluid form were obtained from Egyptian cobras caught in the grounds of the Institute at Onderstepoort. For electrophoresis the venoms were made up as 1% solutions in buffer at pH 6·2 containing 0·0224 M-KH_2PO_4, 0·0036 M-Na_2HPO_4 and 0·044 M-NaCl.

Technique used for the electrophoresis. This has been described in detail by Polson (1945); a brief description of the method follows.

The electrophoresis apparatus and technique used in the present work are slight modifications of those developed by Tiselius (1937). The U-shaped electrophoresis cell used by Tiselius can be cut into segments to facilitate the separation of the components from a protein mixture. This type of cell requires a special arrangement of pistons to shift the segments. For this reason, a less complicated electrophoresis cell was designed (see Figs. 1, 2) which was found suitable for analytical as well as for preparative work. It consists of a U-tube with an inlet tap in the bend and has two side arms of the same diameter fused to the limbs. The two side tubes are attached to two electrode vessels. The ends of the U-tube are open so that samples may be withdrawn from the protein column in either limb.

A fine pipette with its point bent at right angles is used for taking samples. The movement of the pipette in and out of the U-tube is effected by a microscope rack and pinion. The rack and pinion are fixed to the cell holder just above the open ends of the U-tube. The cell holder is attached to an X-ray tube stand with which it can be lowered into and lifted out of the thermostat without disturbing the fluid in the electrophoresis cell.

A typical electrophoresis run on a venom is made as follows: The temperature of the thermostat is lowered to 2° and the cell filled with buffer solution. The silver chloride electrodes are lowered into their respective electrode vessels. These are then covered with saturated KCl solution and the protein inlet tube is fixed on to the capillary tap in the bend of the U-tube. The cell is then lowered into the thermostat, and when temperature equilibrium is attained the protein solution is run in to a level where the column can be photographed. To make an initial sharp boundary, which is essential in electrophoresis, the boundary is rectified by lowering the fine pipette into the U-tube to touch the protein level. A small quantity of fluid is then drawn off and a very sharp interface formed. The current is then switched on and passed through the apparatus until the required separation of components is effected. Using the Lamm (1928, 1937) scale method for observing the migration of the components, a photograph of the scale is taken. Samples are then drawn off at a fixed position in one of the limbs of the U-tube. After each sample is removed a photograph of the scale is taken. Each position where a sample was taken is indicated by a blurred region on the photograph of the scale. In this way the whole of the protein column in a limb is cut up into small samples. The electrophoresis current is now reversed and passed through the cell for the same period as before and the whole process of sampling and photographing repeated.

Determination of toxicity of venom fractions. The toxicity of each sample was tested by the intravenous injection of serial two-fold dilutions into mice, the volume of dose being 0·2 ml. The haemolysin content (Ganguly, 1937) was also determined by tests using 2% suspensions of washed guinea-pig red cells. In these tests it was found that complete haemolysis of all mixtures occurred on standing overnight in the water-bath; readings were therefore taken at the end of 20 min.

The activities of the samples were noted on the electrophoresis diagrams at the positions where they were taken; the position of the toxic component was taken as the peak in the region where the activities of the samples increase most rapidly. A component is considered the active one only if the activity in the ascending as well as the descending column is allocated to the same component.

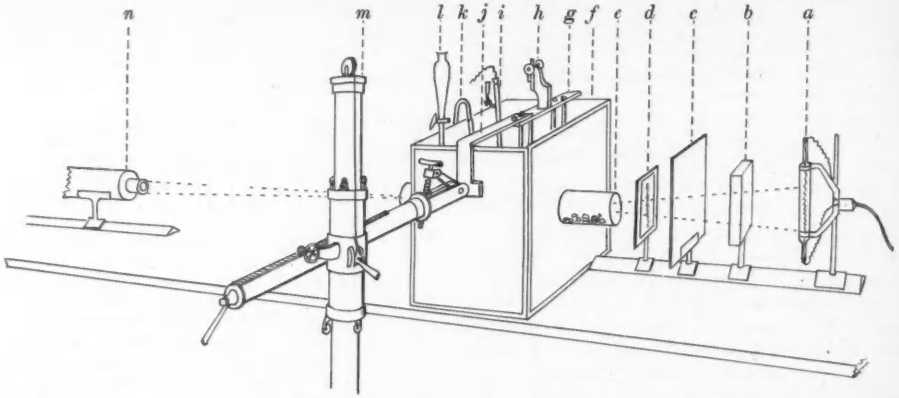

Fig. 1. Electrophoresis apparatus with the cell lowered into the thermostat. *a*, source of illumination (mercury arc); *b*, water filter; *c*, light filter; *d*, vertical transparent scale; *e*, calcium chloride chamber; *f*, thermostat; *g*, tube holder; *h*, microscope rack and pinion; *i*, contact thermometer; *j*, bracket attached to cell holder; *k*, water-stirrer in thermostat; *l*, protein container; *m*, X-ray tube stand; *n*, camera.

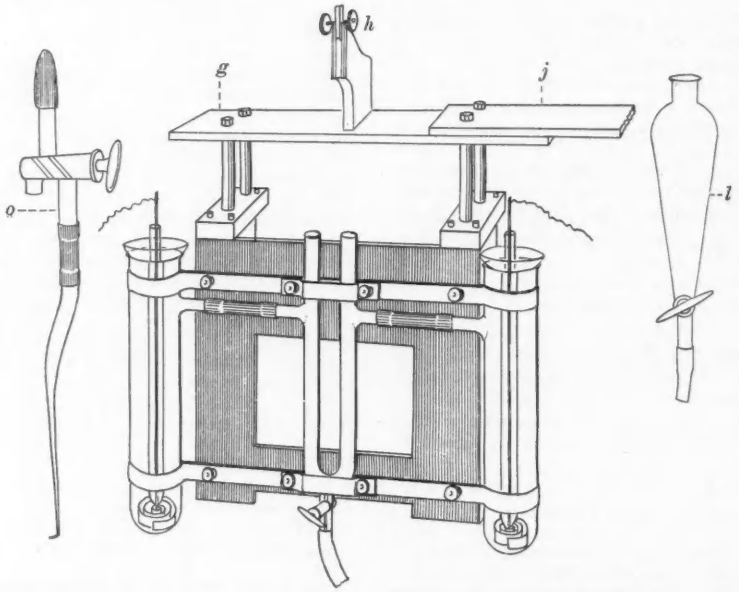

Fig. 2. Electrophoresis cell fixed to holder. *g*, cell holder; *h*, microscope rack and pinion; *j*, bracket attached to X-ray tube holder and cell holder; *l*, protein container attached to U-tube; *o*, sampling pipette.

RESULTS

In Fig. 3 are shown the diagrams of the ascending (A) and descending (B) columns respectively after electrophoresis of *Naja nigricollis* venom at pH 6·2. The diagrams 1–9 in the ascending column (Fig. 3A) and 1–7 in the descending column (Fig. 3B) show the relation of the samples taken to the other components.

From Table 1 it is seen that the toxic and haemolytic component in the ascending column is absent in sample 1 but is present in sample 2. In

the descending column the toxic and haemolytic activity begins with sample 5. When these results are correlated with those of Fig. 3, the activity of the venom can be ascribed to component *T*.

column, and $6{\cdot}2 \times 10^{-5}$ cm.²/sec./V. for the descending column.

In Figs. 4–8 are given the electrophoretic diagrams of other venoms examined. The haemolytic

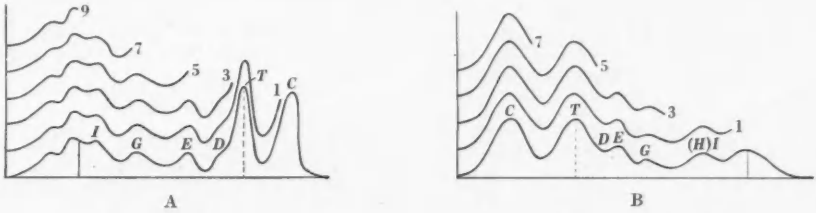

Fig. 3. Electrophoresis diagrams of *Naja nigricollis* venom. A, ascending column; B, descending column; *C*, *T*, *D*, *E*, *G*, (*H*), *I*, various components in original diagram; 1, 3, 5, etc., diagrams of protein column after removal of samples.

Table 1. Naja nigricollis. *Mouse toxicity and haemolytic activity of electrophoretic samples*

Position of component	No. of sample	Dilution 1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Ascending column									
C	1	–°–	–°–	–°–	–°–	–°–	–°–	–°–	– –
T	2	H+H	H+H	H+H	H° –	–°–	–°–	–°–	– –
D	3	H+H	H+H	H+H	H+H	H°H	°	°	– –
	4	H+H	H+H	H+H	H+H	H+H	H°H	H°H	– –
E	5	H+H	H+H	H+H	H+H	H+H	H°H	H°H	– –
	6	H+H	H+H	H+H	H+H	+	H°H	H H	– –
G	7	H+H	H+H	H+H	H+H	+	H°H	H H	– –
	8	H+H	H+H	H+H	H+H	H+H	H°H	H H	– –
I	9	H+H	H+H	H+H	H+H	H+H	H°H	H H	– –
Descending column									
I	1	–°–	–°–	–°–	– –	– –	– –	– –	
	2	–°–	–°–	–°–	– –	– –	– –	– –	
	3	–°–	–°–	–°–	– –	– –	– –	– –	
G	4	–°–	–°–	–°–	– –	– –	– –	– –	
D	5	H+H	H+H	H°H	H°H	–°–	– –	– –	
T	6	H+H	H+H	H+H	H°H	H°H	– –	– –	
	7	H+H	H+H	H+H	H+H	H°H	H H	– –	
C	8	H+H	H+H	H+H	H+H	H°H	H H	H –	
	9	H+H	H+H	H+H	H+H	H°H	H H	H –	

H H complete haemolysis H – partial haemolysis – – no haemolysis
+ lethal for mice ° mice survived.

Applying the formula of Tiselius (1930), the mobility of component *T* is calculated as $6{\cdot}21 \times 10^{-5}$ cm.²/sec./V. at pH 6·2 and 2° for the ascending

and toxic properties of the various components of *N. naja* venom were not examined, but in both the ascending and descending columns of *N. flava* and

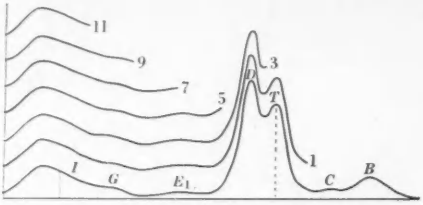

Fig. 4. *Naja flava* venom. Ascending column.

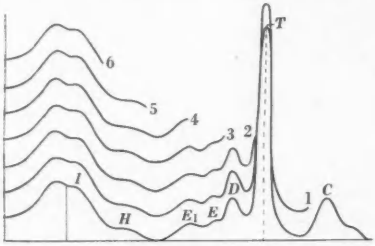

Fig. 5. *Naja haja* venom. Ascending column.

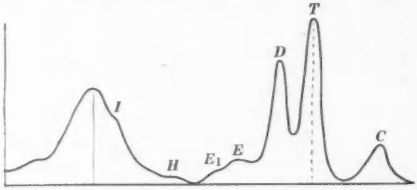

Fig. 6. *Naja haja* (var. *angulosa*) venom. Ascending column.

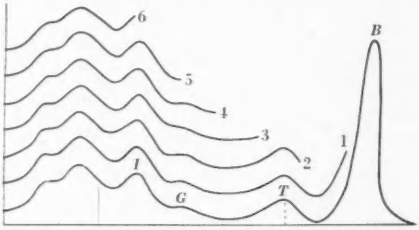

Fig. 7. *Sepedon haemachates* venom. Ascending column.

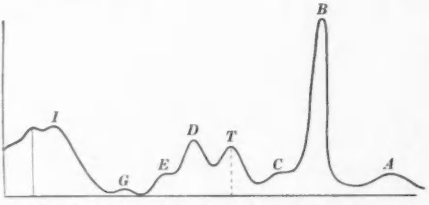

Fig. 8. *Naja naja* venom. Ascending column.

Figs. 4–8. Letters indicate various components in original diagram; numbers indicate diagrams of protein column after removal of samples.

N. haja and in the ascending column of *Sepedon haemachates* these activities were associated, as in the case of *Naja nigricollis*, with component *T*. By analogy, the *T* component of *N. naja* venom was accepted as being the active component.

Electrophoretic characteristics of cobra venoms. In Table 2 are shown the calculated mobilities of the chief components of the various venoms, the calculations being made from the diagrams depicted.

DISCUSSION

At the outset it must be pointed out that the conditions under which the electrophoresis experiments were carried out were identical for each venom, so that all the diagrams are strictly comparable. Consideration of Table 2 shows that the components of the different venoms may be tabulated into groups possessing the same mobility. Two components are common to all the venoms, the non-toxic component *I* and component *T* with which is associated the neurotoxins and haemolysins. This is in accordance with the observations of Grasset (1936) based on *in vitro* and *in vivo* neutralization tests, namely, that there is a common antigenicity between venoms of different species and genera of colubride reptiles.

Further observations by Grasset were that the anavenom (toxoid) prepared from the venom of *Sepedon haemachates* confers upon an animal a satisfactory degree of immunity to the homologous venom but a much less marked immunity to other venoms of the *Naja* and *Dendrapsis* groups, while anti-*Naja flava* serum has approximately the same neutralizing effect upon the various colubride venoms of its own group as upon those of *Sepedon* and *Dendrapsis*. For these and other reasons, notably one of availability, *Naja flava* venom was chosen as the group anti-cobra antigen for the preparation of South African polyvalent serum at the South African Institute for Medical Research.

Consideration of Table 2 in conjunction with the respective electrophoretic diagrams brings out several additional points of interest. *Sepedon haemachates* venom is the least complex, since only four well-defined components were detectable. For this reason it might be anticipated that it would prove to be a good antigen owing to the relatively small number of non-specific protein components present. Actually it was found to be the least efficient of the antigens investigated (Grasset, 1936), a fact which could be ascribed to the exceedingly limited size of the *T* component in the diagram (i.e. to a relatively high proportion of non-specific protein). This conclusion is supported by the finding that in spite of its greater complexity (six well-defined components), *Naja flava* venom proved to be a superior antigen and in the diagram shows a larger *T* component. If the antigenicity of a venom is a function of the

Table 2. *Mobilities of the components of various cobra venoms*

(Expressed as 10^{-5} cm.^{2}/sec./V. at pH 6·2 and 2°.)

Cobra	Components									
	A	*B*	*C*	*T*	*D*	*E*	*F*	*G*	*H*	*I*
Naja nigricollis	—	—	8·02	6·21	5·24	4·12	—	—	2·11	0·63
Naja haja (var. *angulosa*	—	—	8·5	6·40	5·4	4·0	3·6	—	2·1	0·3
Naja haja	—	—	8·6	6·6	5·6	4·4	3·6	—	2·07	0·65
Naja naja	11·7	9·3	8·1	6·52	5·3	4·3	—	3·01	—	0·74
Naja flava	—	9·00	8·0	6·44	5·6	—	3·6	—	—	1·1
Sepedon haemachates	—	9·3	—	6·46	—	—	—	2·94	—	1·4

mass of the *T* component shown in the electrophoretic diagram, then *N. nigricollis* venom should be only slightly inferior to *N. flava*. *N. naja* venom should be at best only slightly superior to *Sepedon haemachates* venom and markedly inferior to the others. It would be of interest to show whether *Naja naja* venom is superior as an antigen to *N. flava* or whether the greater mass of the *T* component is counterbalanced by the greater complexity as shown by the presence of an additional well-marked component.

SUMMARY

1. The electrophoresis of a number of cobra venoms is described, together with the method of identification of the toxic component in the electrophoresis diagram.

2. The venoms of *Naja nigricollis*, *N. haja*, *N. naja*, *N. flava* and *Sepedon haemachates* were examined electrophoretically, and the respective diagrams obtained under identical conditions at pH 6·2 are shown.

3. The neurotoxic and haemolytic activity is found to be associated with the *T* component having a mobility of about $6{\cdot}4 \times 10^{-5}$ cm.^{2}/sec./V.

4. The various components of the different venoms fall into groups having the same mobility.

5. *Sepedon haemachates* venom is the least complex and *Naja naja* venom the most complex.

6. The contention that the efficiency of an antigen may be gauged by the mass of the *T* component is discussed.

One of us (F.J.J.) expresses his gratitude to the Director, Dr P. J. du Toit, for the opportunity of working at his Institute. We are indebted to the South African Institute for Medical Research, Johannesburg, for the supply of desiccated venoms.

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The Metabolism and Functioning of Vitamin-like Compounds

2. A COMPARISON OF PANTOTHENATE METABOLISM BY PROLIFERATING AND BY NON-PROLIFERATING BACTERIA

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Suspensions of non-proliferating haemolytic streptococci were previously found (McIlwain & Hughes, 1944) to destroy the growth-promoting powers of pantothenate, for streptococci and for other organisms, during glycolysis. Such pantothenate inactivation was inhibited by pantoyltaurine, which inhibited also the growth of streptococci; the activities of pantoyltaurine in these two respects towards a series of organisms were quantitatively correlated, as were also the effects of a series of compounds structurally similar to pantoyltaurine, upon a given organism (McIlwain & Hughes, 1945). Preformed

pantothenate was necessary for the growth of the organisms concerned, and a knowledge of the manner of connexion between its participation in the two processes of metabolism and growth might give knowledge of reactions underlying the biological functioning of a vitamin-like substance.

The present paper examines an hypothesis concerning this connexion, based on the following argument. Pantothenate is normally assimilated during the growth of haemolytic streptococci, but the possibility of such assimilation is limited in non-proliferating suspensions of the organisms; reactions which in growing cultures led to assimilation of pantothenate might then, in non-proliferating cells, lead to catabolism of pantothenate through lack of co-ordination between pantothenate metabolism and reactions which in growing organisms result in the synthesis of new bacterial substance. This suggestion led to the expectation that pantothenate inactivation would not occur to the same extent in growing cultures as in suspensions of non-proliferating organisms. It appeared especially desirable to find whether this was the case, as few comparable studies of the metabolism of growth essentials in growing and non-growing cells have been encountered. Though the 'resting-cell' technique is widely applicable to studies of microbial metabolism, vitamin-like substances, through their special connexion with growth, may well be in a special category in this respect; certain of their reactions cannot occur without concomitant growth. Also, an assessment of bacterial economy demands an understanding of the inactivation of pantothenate by streptococci, for in a given environment this activity would appear to restrict their potential growth.

Streptococcal inactivation of pantothenate, in concentrations comparable to those of natural environments of the organisms, has been found nearly proportional to time and expressed as a metabolic quotient Q_P (in mμ mol./mg. dry wt./hr.) for comparison with the reaction as it occurred during growth. To obtain this latter value the pantothenate present initially and at a few intervals during growth was determined, and the bacterial activity with which the changes were associated was obtained by graphical integration of the growth curve of the culture. The activity was expressed in mg.-hr. (the product of the dry weight of bacteria in mg., and the time in hr. during which they were acting). The growth curve was obtained from optical density measurements of samples taken from the culture during growth; a logarithmic plot of the optical density was made to characterize the different phases of growth of the culture, whose pantothenate metabolism were in a few cases compared. To approach the wider problems indicated above, the scope of the investigation has been extended by applying similar methods to strains of *Proteus morganii*, whose inactivation of pantothenate was more rapid than that of the streptococci, and also to *Escherichia coli* and *Pseudomonas aeruginosa* (*Ps. pyocyanea*) which synthesized pantothenate.

EXPERIMENTAL

Organisms. The following bacterial strains were examined: (R), the 'Richards' strain of *Streptococcus haemolyticus*, No. 5631 of the National Collection of Type Cultures, passaged in mice before and during the investigations; (G), a β-haemolytic streptococcus of group G; (F) a *Streptococcus faecalis*, isolated from human faeces, being strain II of McIlwain & Hughes (1945); two strains of *Proteus morganii*, N.C.T.C. no. 2818 and strain 2 of McIlwain & Hughes (1945); *Pseudomonas aeruginosa* (strains 3 and 4) isolated from an infected wound and given by Dr L. Colebrook; and two recently isolated strains of *Esch. coli* from faeces.

The β-haemolytic streptococci were maintained, after passage, by weekly subculture in blood broth, from which subcultures to serum-broth-agar slopes were made for daily use. Other organisms were subcultured on broth-agar slopes.

Growth media for streptococci. The *casein medium* of Table 1 contained a hydrolysate of casein prepared by acid in the presence of titanium salts, glucose, phosphate, and small quantities of many growth-promoting substances. It was prepared by adding aseptically to the basal medium (70 ml.) of McIlwain & Hughes (1944), M-glucose (5 ml.); 0·02M-arginine and 0·02M-glutamine (2 ml.); 0·5M-sodium bicarbonate (0·2 ml.); group A of McIlwain (1944) (5 ml., containing $\frac{1}{25}$ of the concentration of Fe salts there described); N-NaOH to neutralize the acid of group A (1 ml.) pantothenate as required; an inoculum containing *c.* 10^7 organisms in 0·1 ml. of the medium itself, and water to 100 ml. *Casein-yeast* and *casein-broth* media were prepared in the same manner as the casein medium except that the pantothenate-free yeast preparation of McIlwain (1944) was added (1 ml./100 ml.) in the former case and an ordinary bacteriological infusion broth (10 ml.) in the latter, in place of equal volumes of water.

Growth media for Pr. morganii. The casein medium was the 'assay medium' of McIlwain & Hughes (1945) with added pantothenate and prepared from the same reagents as for streptococci but containing fewer accessory factors; the *casein-yeast* medium was again prepared from it by addition of 1% of the pantothenate-free yeast preparation.

Ps. aeruginosa was grown in the ammonium lactate and inorganic salts of Fildes (1940); for *Esch. coli* either this medium, or one in which glucose (final concentration, M/80) replaced the lactate, was used.

Pantothenate metabolism during growth

Media were prepared in volumes slightly in excess of those of the intended cultures (e.g. 320 ml. for three cultures of 100 ml.) and portions of uninoculated media kept for the assay of their original pantothenate concentration, for preparation of the inoculum, and to serve as control in the photometric measurement of subsequent growth. The remainder was inoculated and portions of 100 ml. pipetted to 250 ml. conical flasks which were shaken at about two

oscillations of 5 cm./sec. in a thermostat at 37°. Portions of *c.* 1·5 ml. were taken aseptically at intervals (usually of ½ hr.) during growth by Pasteur pipettes, after manually shaking the flasks. After the desired growth, the cultures were no longer handled aseptically; their volume was measured, a portion taken for determination of the dry weight of bacteria present, the organisms of the bulk collected by centrifuging, and a portion of the culture fluid filtered to sterilize. Its pantothenate content, and that of the organisms after digestion (McIlwain & Hughes, 1944) were determined by growth of *Pr. morganii* (McIlwain & Hughes, 1945). With the streptococci and *Pr. morganii*, the pantothenate of the bacteria was a small fraction only, <1%, of that of the initial solution and values for bacterial pantothenate are recorded separately in only a few cases; the pantothenate content of cultures after growth, in Table 2, include the values for that of the bacterial cells. Change in the pantothenate of the bacterial cell during growth is not considered unimportant, but is being studied separately. Changes in pH during growth under the present conditions were <0·2 pH unit.

Measurement of growth

The optical densities of samples taken during growth were compared with that of the original medium in a Hilger photoelectric absorptiometer. Neutral filters and cells taking 0·5 ml. of liquid were used; the 1·5 ml. samples were adequate for washing a cell between consecutive readings. Significant readings were not obtained from specimens taken earlier than 1 hr. after inoculation, but the initial point of each growth curve was obtained accurately by measuring the optical density of a portion of the suspension used as inoculum; this had been prepared in the medium itself and was diluted to a known extent in inoculation. The course of growth during the initial period could usually be deduced with reasonable certainty from such data (see Fig. 2); the bacterial mg.-hr. during this period was a very small fraction of the whole. Culture fluids were not found to change in light absorption as a result of growth; the use of titanium salts during preparation of the casein hydrolysate gave media with small initial light-absorbing capacities. By examination of a series of dilutions prepared from two bacterial suspensions, in their growth media, the densities given by the instrument were shown to be proportional to the quantity of bacteria present. The ratio between bacterial mass and optical density was determined in each experiment by finding the dry weight of bacteria present in a sample from one of the cultures.

Pantothenate metabolism of bacterial suspensions

The suspensions were of organisms grown in the above manner, and also of some grown in a more normal and convenient bacteriological fashion: in similar media but from smaller inocula, overnight, in an incubator, and without shaking. They were washed twice in 0·5% NaCl by centrifuging, and suspended in this solution for use. Reactions were carried out at 37° and pH 7·6 in Warburg vessels of *c.* 20 ml., usually containing the reactants (Table 1; cf. also McIlwain & Hughes, 1945) in 3·3 ml., and lasting ½–2 hr. The reactions of streptococci and *Pr. morganii* towards pantothenate, in the presence of excess glucose, were found to be independent of oxygen and most experiments with these organisms were performed anaerobically, in a N_2-CO_2 atmosphere forming a buffer with $NaHCO_3$ in solution. Those with *Esch. coli* and *Ps. aeruginosa* were carried out aerobically, as were the growth experiments, and in phosphate buffer.

Pantothenate before and after metabolism was determined as described above. The response of the assay organism to pantothenate has already been found (McIlwain & Hughes, 1945) to be independent of various constituents present during the metabolic experiments; in addition to control experiments described below, the assay of small quantities of pantothenate in the presence of β-alanine and pantoic acid was examined, and found to be unaffected by them.

RESULTS

β-Haemolytic streptococci as non-proliferating suspensions

Comparison of the metabolism of pantothenate by growing and non-growing bacteria necessitated the use of different media in the two cases, if normal organisms were to be used and in one case they were not to grow. Also, the composition of a medium would change during growth. To find whether significant values could be obtained for pantothenate metabolism under such circumstances, the effects of different constituents of the growth media and of other variable factors on the reaction were first examined. Excess of glucose was added in all cases as such addition was necessary both for growth and for pantothenate metabolism in the absence of growth.

(1) *Pantothenate concentration and time of reaction.* Fig. 1A shows the progress of pantothenate inactivation by the 'Richards' strain of streptococcus to be roughly proportional to time, but to fall slightly in rate as the reaction progressed. The first three points correspond to a value for Q_P of $-1{\cdot}31$ and the last three, $-1{\cdot}16$. Similar results with the group G streptococcus have been given previously (McIlwain, 1944). The range of pantothenate concentrations in these experiments was relatively large, 0·3–6·6, and $0{\cdot}4$–$2{\cdot}2 \times 10^{-6}$ M, and covered those of most animal tissues and of the present experimental media. The time occupied by the present experiments with streptococcal suspensions was not greater than that of Fig. 1A. It is therefore considered that the rate of pantothenate metabolism by the present suspensions is sufficiently stable to be evaluated as a mean Q_P over periods of about an hour.

(2) *Bacterial suspensions.* Table 1 shows the largest variation in Q_P of a given streptococcal strain to be associated with the growth and handling of the suspension examined. Q_P varied little with age of suspension until cultures were long past their period of maximal growth rate. Organisms collected at 5 hr. when growth was rapid and the bacterial mass was about one-fifth of the maximum obtainable in the medium, were found to be of

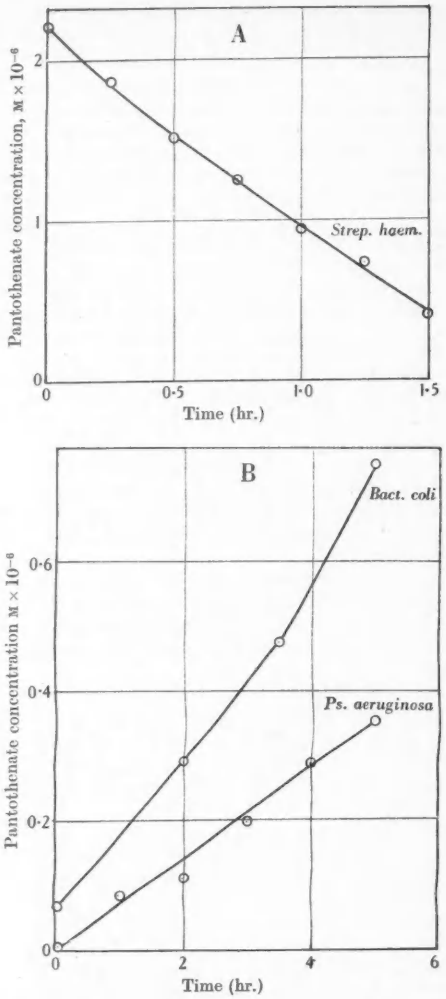

Fig. 1. Kinetics of pantothenate metabolism by bacterial suspensions. (A) Inactivation by the 'Richards' streptococcus. Experimental procedure as described by McIlwain & Hughes (1945). The organisms (3·5 mg. dry wt.; parent culture, 24 hr. old) were added to glucose-bicarbonate-saline (final volume 3·5 ml.) and gave a mean value for Q_P of −1·25. (B) Pantothenate production. *Esch. coli* (3·35 mg. dry wt.; parent culture, 24 hr. old) was suspended in 25 ml. glucose-phosphate-saline; Q_P during the first 3½ hr., +0·88. A 6% increase in optical density occurred during the latter part of the experiment. *Ps. aeruginosa* (10·3 mg. dry wt.; strain 3, parent culture 7 hr. old) was suspended in 100 ml. lactate-phosphate-saline. No growth, but a 5% diminution in optical density occurred during the experiment. Mean Q_P: +0·70.

approximately the same Q_P as organisms collected at 18 hr., when the cells had been metabolizing for some time without much increase in bacterial substance. Between 18 and 24 hr. both glycolyzing activity and Q_P fell. Harvested organisms did not decrease in Q_P if they were kept cold for 2 hr., but Q_P and rate of glycolysis fell after glycolysis in a simple medium and resuspension of the organisms in another portion of the same medium. This fall was more rapid than in the richer media used for growth. The decrease in both cases was presumed to be due to exhaustion of various systems directly or indirectly connected with glycolysis, such as may be illustrated by the effect of glutamine studied previously (McIlwain, 1946) with streptococcal strains which included those of Table 1.

(3) *Medium constituents other than glucose and pantothenate.* The effects of such constituents were investigated most fully in the cases of the two β-haemolytic streptococci, and were found to be small. The casein preparation, representing the greater mass and number of substances of any constituent present during growth, caused a small acceleration in pantothenate inactivation. Folic acid and biotin were examined as they had been considered to be involved in pantothenate metabolism in rats (Wright & Welch, 1943; Emerson & Wurtz, 1944). One interpretation of the observations of Wright & Welch (1943) could be that the two compounds decreased pantothenate-inactivation; but their effect upon the reaction in streptococci was negligible. The preparation of folic acid (*L. casei* factor, 80% pure) which was used was that of Hutchings, Stokstad, Bohonos & Slobodkin (1944) and I am greatly indebted to Dr E. L. R. Stokstad for providing it. Sevag & Green's (1944) opinion that a function of pantothenate in staphylococci was in the synthesis of tryptophan suggested that pantothenate-usage might be altered by the presence of excess tryptophan; but little effect of tryptophan on streptococcal inactivation of pantothenate was found. Mg salts and adenosine triphosphate (examined on account of their relationship to glycolysis) and glutathione were also inactive in this respect. The results suggest that no considerable change would be expected in Q_P on account of the differences in media in which proliferating and non-proliferating organisms were examined.

β-Haemolytic streptococci during growth

The changes in pantothenate which were observed during growth of several cultures are recorded in Table 2, together with quantitative descriptions of the course of growth. The derivation of these descriptions from experimental data is illustrated in Fig. 2, which gives the growth curve and logarithmic growth curve of a culture of the group G streptococcus. In this case, growth was initiated in two

Table 1. *Rates of pantothenate metabolism by non-proliferating bacterial suspensions*

Vessels initially contained saline suspensions of the organisms (2–10 mg. dry wt.) in a side arm, and in the main compartment a buffering solution and substrate. Quantities (per vessel) and further details of the reagents, indicated below, are: glucose (present in all experiments with streptococci), 200 μmol.; $NaHCO_3$, 150 μmol.; saline, the mixture of Krebs & Eggleston (1940), with however only 3 μmol. of phosphate; phosphate-saline, the preceding with 80 μmol. of phosphate; casein hydrolysate, that of McIlwain & Hughes (1944) (75 mg.); $MgSO_4$, 0·5 μmol.; adenosine triphosphate and adenylic acid, 0·7 μmol. Experiments with streptococci and *Pr. morganii* (except where indicated) were performed anaerobically in an atmosphere of $N_2 + 5\%$ CO_2 and with yellow phosphorus in a centre well. Those with *Esch. coli* and *Ps. aeruginosa* were aerobic (except where indicated otherwise): gas phase, air; NaOH in a centre well.

Organism; age of culture	Reagents	Pantothenate: Initial (M × 10^{-8})	Pantothenate: Final (M × 10^{-8})	Q_P (mμmol./mg. dry wt./hr.)
Str. haem. (G); 23 hr.	Saline; or phosphate-saline; or saline with $MgSO_4$ and either adenosinetriphosphate or adenylic acid	325	126–130	−1·21
	Casein hydrolysate and saline	325	122	−1·25
Str. haem. (G); 17 hr.	Saline-bicarbonate, anaerobically	585	325	−1·72
	Saline-bicarbonate, aerobically	585	310	−1·84
	Casein hydrolysate with and without glutathione (10 μmol.), anaerobically	585	291	−1·96
Str. haem. (G); 23 hr.	Saline-bicarbonate with or without tryptophan (1, 10 μmol.)	500	351	−1·24
	Saline-bicarbonate with folic acid concentrate (0·01, 0·1 mg.) and biotin (0·1, 0·5 μg.), with or without tryptophan (1, 10 μmol.)	500	366	−1·15
Str. haem. (G); 5½ hr.	Saline-bicarbonate	281	150	−1·62
Str. haem. (R); 22 hr.	Saline-bicarbonate; or phosphate-saline, $MgSO_4$, and adenosine triphosphate	348	252	−2·25
	Saline-bicarbonate and casein hydrolysate	348	250	−2·3
Str. faecalis; 18 hr.	Saline-bicarbonate with or without $MgSO_4$ and adenosine triphosphate	504	354	−1·96
Str. faecalis; 24 hr.	Saline-bicarbonate	627	273	−1·43
Pr. morganii (N.C.T.C. 2818); 16 hr.	Saline-bicarbonate-glucose with or without casein hydrolysate and yeast preparation	340	206	−6·9
Pr. morganii (laboratory strain 2); 4 hr.	Saline-bicarbonate-glucose	336	269	−1·1
	Saline-bicarbonate-glucose with casein hydrolysate and yeast preparation	336	81	−4·1
Esch. coli; 18 hr.	Saline	6·7	12	+0·26
	Saline-glucose	6·7	75	+2·8
	Saline-glucose with β-alanine (10 μmol.); 0–2 hr.	6·7	310	+30·3
	Saline-glucose with β-alanine (10 μmol.); 2–5 hr.	310	940	+43·5
Ps. aeruginosa (strain 3); 40 hr.	Phosphate-saline	0·1	0·28	+0·04
	Phosphate-saline with lactate (200 μmol.)	0·1	4·0	+0·92
Ps. aeruginosa (strain 3); 48 hr.	Phosphate-saline with lactate (200 μmol.)	0·3	2·5	+0·54
	Phosphate-saline with lactate (200 μmol.) and β-alanine (1 μmol.)	0·3	2·65	+0·57
Ps. aeruginosa (strain 3); 48 hr.	Phosphate-saline with lactate (200 μmol.)	0·3	2·72	+0·47
	Phosphate-saline with lactate (200 μmol.) and pantoic acid (10 μmol.)	0·3	8·90	+1·67

portions of the same medium, one culture being continued for 3 hr. and the other for 5 hr. and the initial and final pantothenate of each determined. The logarithmic plot of optical density readings of specimens taken from the cultures at different times, showed the cultures to grow with negligible lag and with two phases of logarithmic increase. The rate of growth during these phases has been described in Table 2 by their slope, expressed as a mean generation time (m.g.t.; cf. Dagley & Hinshelwood, 1938) or time taken for the readings to double in value. The point of transition from the more rapid to the slower phase was obtained from the logarithmic curve, and this time, together with the

arbitrary time at which growth of the first culture was stopped, divided the bacterial activity into the three parts indicated by different shading in Fig. 2. The shaded areas were measured and the values converted to mg.-hr. by a factor derived from the optical density and dry weight of a measured sample of the second culture. The disappearance of pantothenate from the first culture is thus associated

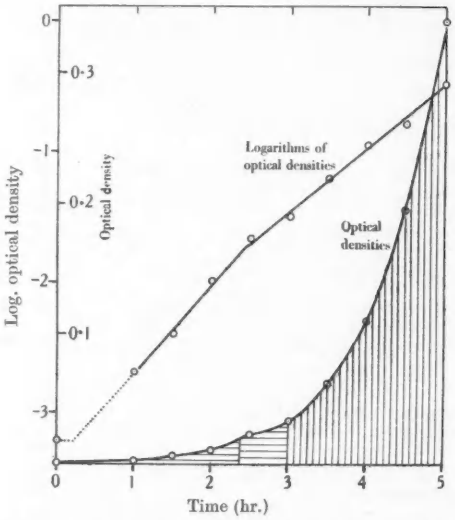

Fig. 2. Growth curve and logarithmic growth curve of the group G streptococcus in the casein-yeast medium, shaken aerobically in a thermostat at 37°. Inocula were added to portions of the warmed medium at a time taken as zero in the figure. Of duplicate cultures, one was removed at 3 hr. for determination of its pantothenate content and the other at 5 hr. Data derived from this figure are given in the asterisked experiment of Table 2; the mg.-hr. of bacterial activity performed in the first culture was proportional to the horizontally shaded area (comprising two portions differing in mean generation time), and the further activity in the second culture, to the vertically shaded area. The mean generation times of the culture were obtained from the slopes of the logarithmic plot; the dotted extrapolation of this indicates the method of computing the length of the lag phase. When this method gave values for the lag phase of <15 min., as in the figure, the existence of this phase was not considered sufficiently well defined to record in Table 2.

partly with growth of m.g.t. 28 min. and partly with that of m.g.t. 38·4 min.; it was small, and was not determined accurately. The further disappearance of pantothenate found to have occurred in the second flask was associated entirely with growth at the slower rate, but with much greater bacterial activity; the greater pantothenate change was determined more accurately.

The rate of pantothenate inactivation during growth is unmistakably of the same order of magnitude as that of non-proliferating suspensions of the organism. A more accurate value for Q_P during the early phase of growth has been obtained in a separate experiment by using a lower initial concentration of pantothenate (Table 2). Further, the Q_P of organisms after growth has been compared with that of the same organisms, during the period of growth immediately preceding their harvesting, with the following result. The group G organism, grown in a casein medium, gave a m.g.t. of 39 min. and Q_P of $-1{\cdot}43$; in a casein medium and without growth, a Q_P of $-1{\cdot}15$ was found. Different determinations of Q_P during growth in different media have given values associated with growth both slower and quicker than that of Fig. 1, but all values for Q_P have fallen into a relatively narrow range. Tables 1 and 2 show the behaviour of the 'Richards' streptococcus to be similar.

Other organisms inactivating pantothenate

The behaviour of a strain of *Str. faecalis* towards pantothenate was closely similar to that of the β-haemolytic streptococci (Tables 1, 2). Values of Q_P during growth fell within the same range and were lower during slower growth. The values in non-proliferating suspensions approximated to those during growth.

Proteus morganii inactivated pantothenate during growth at rates notably greater than those of the preceding organisms. Of two strains, one attained a high rate of glycolysis (Q_{CO_2}, 450–650 μl./mg. dry wt./hr.) and a rate of pantothenate metabolism approximating to that in culture, when metabolizing in a relatively simple medium. Glycolysis of the other strain was slow (Q_{CO_2}, *c.* 150) in a simple medium, and its rate of inactivation of pantothenate was also much lower than in culture. The rates of both processes were increased by addition of the casein and yeast preparations present during growth, and the Q_P then approximated to that found to accompany growth. The addenda did not include all substances used in growth of the organisms, and growth did not occur during the metabolic experiment. In an experiment in which the rate of pantothenate inactivation during growth was compared with that given by the same organisms immediately afterwards, as a non-proliferating suspension, Q_P during growth of m.g.t. 42 min. in casein-marmite-glucose was $-3{\cdot}8$ and of the separated organisms, $-4{\cdot}1$ mμmol./mg. dry wt./hr.

Organisms synthesizing pantothenate

Esch. coli. Growth of the present strain in the medium of glucose and inorganic salts was not obviously affected by addition of 10^{-5} or 10^{-7} M-pantothenate, but this substance was found to be

Table 2. *Rates of pantothenate metabolism during growth of bacteria*

One or more flasks containing 100 ml. of the media indicated were inoculated, and specimens taken at intervals to give growth curves such as that of Fig. 2, from which were obtained the data of the second column of the table. The asterisked experiment is that of Fig. 2. Solution and cells were separated after growth and the pantothenate of each determined. In a few cases in which the pantothenate change was a small fraction of that present, the large possible error is noted.

	Phases of growth			Pantothenate		
Organism; medium of growth	Description (m.g.t. in min.)	Duration (min.)	Bacterial activity (mg.-hr.)	Initial (mμmol.)	Final (mμmol.)	Q_P (mμmol./mg. dry wt./hr.)
Str. haem. (G); casein hydrolysate	log., m.g.t. 45 log., m.g.t. 62	270 210	40·0	100	38	−1·55
Str. haem. (G); casein hydrolysate + yeast preparation	lag log., m.g.t. 29 log., m.g.t. 60	90 270 90	34	100	20	−2·35
Str. haem. (G)*; casein hydrolysate + yeast preparation	log., m.g.t. 26 log., m.g.t. 37 log., m.g.t. 37	144 36 120	1·62 1·92 32·5	94 87	87 50	−2·0 (±0·5) −1·14
Str. haem. (G); casein hydrolysate + broth	log., m.g.t. 29 log., m.g.t. 29 log., m.g.t. 57	270 27 63	4·08 4·7 17·7	120 108	108 33	−3·0 (±0·5) −3·35
Str. haem. (G); casein hydrolysate + yeast preparation	lag log., m.g.t. 24	65 135	2·08	9·0	3·3	−2·7
Str. haem. (R); casein hydrolysate + broth	log., m.g.t. 41 log., m.g.t. 87 log., m.g.t. 87	277 81 180	6·0 19	109 96	96 53	−2·1 (±0·3) −2·3
Str. faecalis; casein hydrolysate + broth	log., m.g.t. 25 log., m.g.t. 35 log., m.g.t. 35 Growth slowing to m.g.t. 65	120 120 20 40	10·2 19·8	86 58	58 26	−2·7 −1·6
Pr. morganii (2818); casein hydrolysate + yeast preparation	log., m.g.t. 25·5	180	6·34	100	62	−6·0
Pr. morganii (2818); casein hydrolysate + yeast preparation	log., m.g.t. 28·5 log., m.g.t. 42 log., m.g.t. 42	175 15 60	1·64 4·5	30 23	23 6	−4·3 −3·8
Esch. coli; glucose-inorganic salts	log., m.g.t. 44 log., m.g.t. 56 Gradual retardation from m.g.t. 56 to 210	330 90	16·6 38·6	0·05 25·6	25·6 89·4	+1·54 +1·65
Esch. coli; glucose-inorganic salts	log., m.g.t. 61 log., m.g.t. 61	240 80	0·96 9·8	0·2 7·1	7·1 22·1	+7·0 +1·53
Ps. aeruginosa, strain (4); lactate-inorganic salts	lag log., m.g.t. 37 log., m.g.t. 37	85 125 90	5·1 12·9	0 6·1	6·1 25·0	+1·20 +1·46
Ps. aeruginosa, strain (3); casein hydrolysate	log., m.g.t. 34·5 log., m.g.t. 58	285 75	8·5	0	5·9	+0·70
Ps. aeruginosa, strain (3); lactate-inorganic salts	lag log., m.g.t. 30 log., m.g.t. 30 log., m.g.t. 108	60 180 62 118	1·84 45·5	0 11·2	11·2 44·5	+6·1 +0·73

present in the cells and solution after growth in pantothenate-free media. Suspensions of cells which had been washed twice in 0·5 % NaCl grew little if at all during 3 hr. in a mixture of glucose and inorganic salts lacking NH_4^+ ions. Under these conditions, pantothenate was produced at a steady rate (Fig. 1) which corresponded to a Q_P of +2 to +3 mμmol./mg. dry wt./hr. Without glucose, pantothenate production was much slower. Under either of these conditions, the organisms were presumably supplying a nitrogenous compound for synthesis of pantothenate; when β-alanine was

added as well as glucose, Q_P was greatly increased, to values of +30 or +40 mμmol./mg. dry wt./hr.

Values for Q_P during growth of *Esch. coli* in the glucose-inorganic salt medium varied between +1·5 and +2 while the bulk of growth was taking place, and thus approximated to the rate of the reaction caused by non-proliferating suspensions in comparable media. The values were not markedly affected by rate of growth. In several instances, however (and of these Table 2 gives an example), Q_P during the production of the first few mμmol. of pantothenate was found unusually high. This did not represent carry-over of pantothenate from the previous culture used as inoculum; the inoculum was not washed but its pantothenate (in cells and solution) was estimated and included in the value quoted for the initial pantothenate of the experimental culture. The high rate of production could be due to transfer from the previous culture of substances other than pantothenate; Q_P remained below that observed in the presence of β-alanine. The proportion of the total pantothenate which was present in the bacterial cells varied considerably, from 3 to 12 %. The quantity per unit weight of cell was also variable, values of 0·07, 0·09, 0·15 and 0·42 mμmol./mg. dry wt. having been obtained with two strains in cultures of varying age. The high initial rate of pantothenate production was not associated with an unusual distribution of the substance between cells and solution.

Pantothenate was also produced by strains of *Esch. coli* in solutions of lactate and inorganic salts, but growth in ammonium lactate media followed a lag of some hours, or required large inocula for prompter growth, which made conditions less suitable for comparisons of the present type than those employing glucose.

Pseudomonas aeruginosa. Results with strains of this organism resembled those with *Esch. coli* but since a lactate medium supported a good growth, the majority of experiments were carried out with lactate as carbon source. Pantothenate production by washed cells in lactate and inorganic salts was approximately proportional to time (Fig. 1) and corresponded in different experiments to Q_P values of +0·5 to +1. The production was very slow in absence of lactate and in its presence was not much affected by β-alanine; pantoic acid however increased it.

During growth of the bulk of the organisms in media of ammonium lactate + inorganic salts, the rates of pantothenate production approximated to those found in non-proliferating suspensions. Thus a value for Q_P of +0·72 was obtained with suspensions of organisms (strain 4), immediately after growth, which had been associated with Q_P of +0·95; with strain (3) the corresponding values were +0·70 and +0·73. Again, however, the first few mμmol. of pantothenate were produced at a greater rate.

DISCUSSION

The commonest method of expressing relationships between micro-organisms and vitamin-like substances which they need, preformed, for their growth, has been to state a minimum quantity or concentration of the substance which is found to be needed for good or optimum growth; this may be amplified by determining the quantity of organisms produced with varying quantity of the growth essential. In the case of β-haemolytic streptococci the smallest concentration of pantothenate for optimal growth is about 10^{-7}M. In artificial media such as those of the present investigation, pantothenate concentrations between 10^{-7} and 10^{-5}M or more have little influence on the mass of bacterial growth (see, for example, McIlwain, 1944) and the mass obtained is about that produced in tissue fluids or ordinary bacteriological media. These are usually 10^{-6} to 10^{-5}M with respect to pantothenate, and the reaction of the bacteria to concentrations of pantothenate of this order of magnitude therefore reflects more closely their normal relationship to pantothenate, than does their behaviour to limiting quantities of the substance.

It has been shown above that the interaction between haemolytic streptococci and 10^{-6} to 10^{-5}M-pantothenate is a much more vigorous one than would be anticipated from their minimal requirements for the substance: the organisms inactivated in 1 hr. some 10–25 times the pantothenate with which they could be grown, and some 50–100 times the pantothenate present in their cells. The inactivation was independent of most media constituents but required glucose, a substance present in the normal environments of the organism in a host or a laboratory medium. The inactivation was found also to be to a large extent independent of growth; in comparing different phases in the history of a culture, that of most rapid growth tended to be associated with most rapid inactivation of pantothenate, but the inactivation remained at a relatively high level in the absence of any growth. This finding of a large turnover or usage of pantothenate which could not be assessed by determining its quantity in the total bacterial mass at different times, nor by finding the minimal quantity of pantothenate necessary for growth, emphasizes the dynamic aspect of the interaction of organism and vitamin. It has been shown that a similar relationship holds between streptococci and glutamine which they require, preformed, for growth (McIlwain, 1944); data given incidentally and without comment by Thompson (1942, Tables X, XII) suggest that during growth under certain conditions *Aero-*

bacter aerogenes also inactivates considerable quantities of pantothenate. Lwoff & Lwoff (1937) and Morel (1941) concluded that coenzymes I and II were inactivated by *Haemophilus influenzae* and *Proteus vulgaris* during reactions which occurred in growth, and also independently of it; the coenzymes functioned as growth factors for the organisms, though they could be replaced by simpler substances. This type of relationship between micro-organism and vitamin-like substance is thus fairly common. With respect to its origin, the possibility that pantothenate inactivation occurred in resting cells through restraint of their growth while they were in the presence of excess of a single growth factor has been disproved by the present investigation.

Relationships between vitamin-like substances and bacteria of the intestinal flora of animals have been the subject of many studies but have been considered largely in terms of the possible synthesis of vitamins by such organisms. An exception was the observation of Benesch (1945) that nicotinamide could be destroyed by mixed caecal flora under conditions in which *Clostridium welchii* was the predominant organism. The further potentialities of intestinal organisms in this connexion are illustrated by the present findings with *Streptococcus faecalis* and *Proteus morganii*.

Synthesis of vitamin-like substances by micro-organisms has also usually been expressed in terms of the quantities finally found in cultures rather than in terms of the specific activity of the organisms in producing the substances, such as is provided by metabolic coefficients. The latter method has now enabled comparisons to be made between the synthesis of pantothenate by some organisms and its inactivation by others, and also the detection of the unusual activity of *Esch. coli* and *Ps. aeruginosa* towards pantothenate, in the early phases of cultures. Data given by Thompson (1942, Table XI and Fig. 2) for the production of biotin by *Proteus vulgaris* can in part be treated in this manner, and calculation shows the rates of biotin production during the first two periods of 4 hr., when the m.g.t. was approximately 90 min., to be roughly constant (but of unknown absolute value), with a considerable fall in m.g.t. and rate of production of biotin after that period.

Comparison of pantothenate synthesis by proliferating and non-proliferating organisms can be expected on theoretical grounds to be less straightforward than comparison of the inactivation of the substance under the two conditions. Thus the substrates for the synthesis are not known and the overall pantothenate exchange which is measured may well be a balance between pantothenate production and inactivation. Nevertheless, the values of Q_P observed during production of the bulk of the pantothenate in culture were paralleled by the values obtained in non-growing suspensions of organisms, containing as added compounds only glucose or lactate, and inorganic salts. The organisms, or associated materials, were then supplying at least a nitrogenous compound. The higher values for Q_P in earlier phases of growth were approached or exceeded by the addition of compounds which might be expected to be, or to be related to, the substrates of the synthesis, namely, β-alanine and pantoic acid. Evidence that these substances may be precursors of pantothenate in micro-organisms has previously been given by their replacing pantothenate in the growth of certain organisms requiring pantothenate or such a surrogate for optimal growth. The present observations suggest that the substances may be related also to pantothenate production in *Esch. coli* and *Ps. aeruginosa*, whose growth is independent of added pantothenate.

SUMMARY

1. (*a*) Inactivation and synthesis of pantothenate by non-proliferating suspensions of various bacteria proceeded with definite velocities which could satisfactorily be expressed as metabolic quotients, that adopted, Q_P, being mμmol. change in pantothenate/mg. dry wt./hr.

(*b*) Q_P, during the inactivation of pantothenate by haemolytic streptococci which accompanied their glycolysis, was $-1{\cdot}2$ to $-2{\cdot}5$; corresponding values with *Str. faecalis* were $-1{\cdot}5$ to -2 and with *Proteus morganii*, -4 to -7. The values were relatively little affected by the addition of various vitamin-like substances or other materials present in growth-media.

(*c*) Production of pantothenate by *Esch. coli* suspended in solutions of inorganic salts was slow (Q_P, $+0{\cdot}3$) but increased by addition of glucose (Q_P, $+1$ to $+3$) and still further (Q_P, $+30$ to 40) by β-alanine. *Pseudomonas aeruginosa* also gave low values in salt solutions, increased by lactate and by pantoic acid.

2. (*a*) These activities of non-proliferating organisms were compared with the organisms' metabolism of pantothenate during growth. Values for Q_P in this case were calculated from (i) the mg.-hr. of bacterial activity (obtained from growth curves) during different phases of a culture's growth and (ii) the associated changes in pantothenate content of the cultures.

(*b*) Rates of inactivation of pantothenate by the streptococci and by *Pr. morganii* were largely independent of phase of growth, and were close to the values observed in non-proliferating suspensions of the corresponding organisms.

(*c*) Production of pantothenate by *Ps. aeruginosa* and *Esch. coli* was rapid during a short initial phase; during the greater part of the culture's growth, Q_P

values approximated to those observed in non-proliferating suspensions of the organisms in the presence of the lactic acid or glucose present during growth.

3. Pantothenate inactivation by non-proliferating bacteria is thus not induced by an imbalance in metabolism resulting from the presence of a growth factor without opportunities for growth. Pantothenate synthesis may proceed from β-alanine or pantoic acid.

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Pyruvate Oxidation in Pigeon Brain Catalyzed by Fumarate

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Small amounts of C_4-dicarboxylic acids, when added to finely minced or ground animal tissues, respiring *in vitro*, are well known to stimulate the O_2 uptake; the effect was first noted by Szent-Györgyi and his co-workers, using pigeon breast muscle (Gözsy & Szent-Györgyi, 1934; Annau, Banga, Gözsy, Huszak, Laki, Straub & Szent-Györgyi, 1935). It was found to be a characteristic of these acids that, unlike true substrates, they preserved the respiration rate of the tissue instead of raising it above its initial level. Stare & Baumann (1937) showed that the effect was truly catalytic; using pigeon breast muscle, they found that the increased O_2 consumption was greater than could be accounted for by the complete oxidation of the added C_4-dicarboxylic acid. Annau, Banga, Blazso, Brukner, Laki, Straub & Szent-Györgyi (1936) and Annau & Erdös (1939), using rabbit liver and pig kidney, respectively, have demonstrated that catalysis by succinate and fumarate is markedly greater when pyruvate is being oxidized. Other investigations (Banga, Ochoa & Peters, 1939; Krebs & Eggleston, 1940; Smyth, 1940) have shown fumarate catalysis to be active in other tissues and have confirmed its relation to the pyruvate oxidation system.

Although the stimulation by fumarate of the O_2 uptake of tissues respiring in a medium containing pyruvate is now well recognized, it is suprising to find lack of agreement as to whether the rate of pyruvate utilization is also increased. Unfortunately, most determinations of pyruvate disappearance have been made in the presence of malonate and the literature contains few observations in the absence of this inhibitor. In those cases where malonate has been excluded and the rate of pyruvate disappearance estimated in the presence and absence of fumarate, the observations are conflicting. Thus in pigeon brain, Banga *et al.* (1939), in two experiments quoted, found an increased pyruvate utilization in the presence of fumarate. On the other hand, Annau *et al.* (1936), using rabbit liver, consistently found a decreased rate of disappearance of pyruvate when fumarate was present, while Krebs & Eggleston (1940), working with pigeon breast muscle, reported an increased disappearance in one case and a decreased disappearance in another.

The present investigation is a continuation of previous work (Long, 1943) in which the behaviour of a dialyzed preparation of ground pigeon brain, respiring in a medium containing pyruvate, inorganic phosphate and adenine nucleotide, was quantitatively investigated. With this enzyme system, which is essentially the same as that of Banga *et al.* (1939), the effect of fumarate on the oxidation of pyruvate has now been re-examined. The increased O_2 uptake due to fumarate catalysis, first reported by the last mentioned authors, has been amply confirmed, but their further claim to have observed an increased rate of disappearance of pyruvate could not be supported. Indeed, a slightly decreased rate of pyruvate disappearance has always been found,

and it seems reasonable to assume that this is due to the simultaneous oxidation of fumarate to pyruvate, which is known to take place under these conditions (Long, 1945).

In view of this result, much attention has been given to the measurement of the O_2 uptake of the tissue preparation under different conditions; these further experiments have shown that fumarate catalysis can only take place in the presence of inorganic phosphate and adenine nucleotide. The most important conclusion, however, based on a study of the O_2/pyruvate ratio, is that fumarate catalysis acts by causing *complete* oxidation of that amount of pyruvate which is only *partially* oxidized in its absence.

EXPERIMENTAL

Materials and methods

The specimens of Na pyruvate, Na α-ketobutyrate, Na arsenite, fumaric acid and adenine nucleotide, previously described, were used. The preparation of the dialyzed pigeon brain suspensions, the respiration technique using Barcroft manometers and the estimation of pyruvate are also similar to those described in earlier papers (Long, 1942, 1943, 1945).

RESULTS

(1) *The effect of fumarate on the* O_2 *uptake and pyruvate disappearance in a phosphate buffered medium. Adenine nucleotide absent.* The absorption of molecular O_2 by dialyzed pigeon brain suspensions, respiring in a medium containing inorganic phosphate and chloride only, has been found to be increased appreciably by addition of either pyruvate (Long, 1943) or fumarate (Long, 1945). In view of this and the known catalytic effect of fumarate on the O_2 uptake of tissues respiring in pyruvate, in certain circumstances, experiments have been carried out to determine whether fumarate catalysis occurs under the conditions described above. The replacement of pyruvate by α-ketobutyrate, which is similarly oxidized by this enzyme system (Long, 1943), has also been studied. The results obtained are shown in Table 1.

It will be observed that the extra O_2 uptake caused by fumarate is less in the presence of pyruvate or α-ketobutyrate than in the absence of substrate. This can be explained as follows. In the absence of substrate, fumarate is oxidized to pyruvate (Long, 1945) which then undergoes further oxidation; the extra O_2 uptake accompanying these two oxidations is quite appreciable (av. 180 μl./g./hr.). When pyruvate is initially present in the respiration medium, however, its relatively high concentration saturates the pyruvate oxidation system, so that the additional pyruvate formed from fumarate cannot further increase the O_2 uptake. The slightly increased O_2 uptake which is observed (av. 72 μl./g./hr.) is undoubtedly therefore that amount which accompanies the oxidation of fumarate to pyruvate. Support for this explanation is to be found in the extent of pyruvate formation from fumarate under these conditions, as given by Long (1945). Thus in three published experiments the amounts of pyruvate produced in the presence of 0·008 M-arsenite were 4·9, 8·2 and 6·2 μmol./g./hr. The average value of 6·4 μmol. pyruvate must have been derived from an equimolecular amount of fumarate. The O_2 uptake required for the oxidation of this amount of fumarate is also 72 μl./g./hr.

The results obtained using α-ketobutyrate as substrate can be explained by the inhibiting effect of this substance on the further oxidation of pyruvate formed from fumarate, since both α-ketoacids are oxidized by the same enzyme system. This inhibiting effect is well shown by the experiments quoted in Table 2. It will be seen that the extra O_2 uptakes due to pyruvate in the absence of α-ketobutyrate, given by the difference between columns (3) and (1) are 470 and 218 μl./g./hr. respectively; in the presence of α-ketobutyrate they are only 284 and 51 μl./g./hr., as given by the difference between columns (4) and (2). Thus α-ketobutyrate inhibits pyruvate oxidation by from 40 to 80 %.

Referring back to the data in Table 1, it must be concluded that since fumarate does not increase the net O_2 uptake due to pyruvate oxidation, fumarate catalysis is absent under these conditions.

Table 1. *The effect of fumarate on the* O_2 *uptake of dialyzed pigeon brain suspensions, respiring in the presence and absence of pyruvate and α-ketobutyrate*

Pigeon brain suspensions dialyzed for 3 hr. against 0·4 % KCl at 2°. Pyruvate and α-ketobutyrate, final concentration 0·01 M; fumarate, final concentration 0·005 M. Respiration conducted at 28°, pH 7·3. Phosphate buffer (0·05 M) present.

Substrate	O_2 uptake (μl./g. fresh tissue/hr.)								
	None			Pyruvate			α-Ketobutyrate		
Exp.	Control	Fumarate	Diff.	Control	Fumarate	Diff.	Control	Fumarate	Diff.
1	225	387	162	472	560	88	350	387	37
2	186	384	198	514	570	56	402	427	25
Av.	206	386	180	493	565	72	376	407	31

Table 2. *The effect of α-ketobutyrate on pyruvate oxidation*

Experimental conditions as in Table 1.

	O_2 uptake (μl./g./hr.)			
Exp.	(1) No substrate	(2) α-Keto-butyrate	(3) Pyruvate	(4) Pyruvate +α-keto-butyrate
1	500	686	970	970
2	320	509	538	560

The effect of fumarate on the rate of pyruvate disappearance has been determined in two cases (Table 3); less pyruvate is seen to disappear from the respiration medium in the presence of fumarate than in its absence. This result is also explained by the simultaneous formation of pyruvate from fumarate; the presence of excess pyruvate in the medium prevents further oxidation of this amount, since the pyruvate oxidation system is already saturated. This explanation is supported by the fact that the amount of pyruvate formed from fumarate in the presence of 0·008 M-arsenite, which was simultaneously determined in one of these experiments, was found to be almost identical with the difference between the amounts of pyruvate disappearing in the presence and absence of fumarate.

Table 3. *The effect of fumarate on the rate of pyruvate disappearance*

Experimental details as in Table 1.

	Pyruvic acid (μmol./g./hr.)			
Exp.	Dis-appearing in absence of fumarate	Dis-appearing in presence of fumarate	Diff.	Formed from fumarate in presence of arsenite
1	39·0	26·5	12·5	11·8
2	39·7	23·5	16·2	—

(2) *The effect of fumarate on the O_2 uptake and pyruvate disappearance in the presence of inorganic phosphate and adenine nucleotide.* Banga *et al.* (1939) observed a considerable increase in O_2 consumption on adding fumarate to a dialyzed pigeon brain suspension, respiring in a medium containing inorganic phosphate, adenine nucleotide and pyruvate; in control experiments, without pyruvate, the effect of fumarate was small. These observations have been confirmed repeatedly in the present work (Table 4) and are to be contrasted with results previously obtained in the absence of adenine nucleotide (Table 1). The occurrence of fumarate catalysis in brain suspensions seems therefore to be dependent on the presence of inorganic phosphate and adenine nucleotide in the respiration medium. It is interesting to note that pyruvate cannot be replaced by α-ketobutyrate in this system, even under conditions which favour activation of α-ketobutyrate oxidation by added adenine nucleotide, i.e. prolonged (8 hr.) dialysis of the enzyme suspension (Long, 1943).

Using this same enzyme system, Banga *et al.* (1939) have also reported that fumarate catalysis is accompanied by an increased utilization of pyruvate; this observation has not been confirmed in the present work. In Table 5 are quoted the results

Table 5. *The effect of fumarate on the extent of pyruvate disappearance in the presence of inorganic phosphate and adenine nucleotide*

Experimental conditions as in Table 4.

	Pyruvic acid (μmol./g./hr.)			
Exp.	Dis-appearing in absence of fumarate	Dis-appearing in presence of fumarate	Diff.	Formed from fumarate in presence of arsenite
1	30·8	27·0	3·8	2·6
2	36·1	33·2	2·9	4·3
3	42·8	35·4	7·4	10·2
4	37·2	31·3	5·9	8·0
5	42·2	35·6	6·6	8·2
6	52·8	39·0	13·8	10·5
Av.	40·3	33·6	6·7	7·3

obtained in a number of experiments in which the rate of disappearance of pyruvate was determined in the presence and absence of fumarate. In all

Table 4. *The effect of fumarate on the O_2 uptake of pigeon brain suspensions, respiring in pyruvate or α-ketobutyrate; inorganic phosphate and adenine nucleotide present*

Adenine nucleotide, final concentration 0·0003 M. Other experimental conditions as in Table 1.

	O_2 uptake (μl./g./hr.)								
Substrate	None			Pyruvate			α-Ketobutyrate		
Exp.	Control	Fumarate	Diff.	Control	Fumarate	Diff.	Control	Fumarate	Diff.
1	319	634	315	1157	1988	831	—	—	—
2	356	740	384	1350	2100	750	—	—	—
3	345	708	363	1192	1902	710	—	—	—
Av.	340	694	354	1233	1997	764			
4*	115	251	136	—	—	—	271	312	41

* 8 hr. dialysis.

cases the amount of pyruvate disappearing is slightly less in the presence of fumarate than in its absence, and the difference between the two amounts is very nearly equal to the quantity of pyruvate formed from fumarate in the presence of arsenite. Thus it must be concluded that inorganic phosphate-fumarate-adenine nucleotide catalysis, while stimulating the O_2 uptake, is without effect on the rate of pyruvate disappearance.

(3) *The O_2/pyruvate ratio for phosphate-fumarate-adenine nucleotide catalysis of pyruvate oxidation.* Previous work (Long, 1943) on the respiring enzyme system—dialyzed pigeon brain suspension, pyruvate and inorganic phosphate—has shown that addition of a catalytic amount (0·0003 M) of adenine nucleotide (A.N.) increases not only the rates of pyruvate utilization and O_2 uptake, but also the completeness of pyruvate oxidation, as measured by the O_2/pyruvate ratio and R.Q. Since, as was shown in the previous section, fumarate addition increased the extra O_2 uptake due to pyruvate oxidation only when inorganic phosphate and adenine nucleotide were also present in the respiration medium, it became of considerable interest to determine the completeness of pyruvate oxidation when subjected to the combined catalytic actions of phosphate, fumarate and adenine nucleotide. The estimation of the O_2/pyruvate ratio under these conditions has given a satisfactory answer to the problem.

Six duplicate series of Barcroft manometers were taken, each working flask containing 0·05 M-phosphate buffer, pH 7·3, 0·005 M-fumarate and the enzyme preparation. The individual additions to the flasks and their further manipulation were as follows:

Addition	Treatment
(1) None	Allowed to respire for 40 min. after equilibration. O_2 uptake measured.
(2) Pyruvate	Deproteinized after equilibration. Pyruvate content determined.
(3) Pyruvate	Allowed to respire for 40 min. after equilibration, then deproteinized. O_2 uptake and pyruvate content determined.
(4) A.N.	Allowed to respire for 40 min. after equilibration. O_2 uptake measured.
(5) A.N. + pyruvate	Deproteinized after equilibration. Pyruvate content determined.
(6) A.N. + pyruvate	Allowed to respire for 40 min. after equilibration, then deproteinized. O_2 uptake and pyruvate content determined.

In the absence of adenine nucleotide, the difference in O_2 uptake between manometers (3) and (1) gave the extra O_2 uptake due to pyruvate oxidation during the 40 min. respiration period; the difference between the pyruvate contents of flasks (2) and (3) gave the simultaneous pyruvate disappearance. Similarly, for pyruvate oxidation in the presence of adenine nucleotide, the extra O_2 uptake was given by the difference between manometers (6) and (4), and the pyruvate disappearance was equal to the difference in pyruvate content of flasks (5) and (6). By further subtraction of these differences (as shown in Table 6), the net effect of adenine nucleotide on the O_2 uptake and pyruvate disappearance in the presence of phosphate and fumarate was determined, and the ratio of these quantities gave the O_2/pyruvate ratio for pyruvate oxidation activated by combined phosphate-fumarate-adenine nucleotide catalysis. The results obtained in three experiments are shown in Table 6. It should be noted that the method of evaluating the effect of adenine nucleotide on the enzyme system is quite rigid since fumarate oxidation is unaffected by adenine nucleotide (Long, 1945).

Table 6. *The O_2/pyruvate ratio for phosphate-fumarate-adenine nucleotide catalysis of pyruvate oxidation*

For experimental details, see text. The amount of dialyzed brain suspension corresponds to 333 mg. fresh tissue. Respiration period, 40 min. (A.N. = adenine nucleotide.)

	Extra O_2 uptake (μmol.)			Pyruvate disappearance (μmol.)			
Exp.	A.N. absent (3)–(1)	A.N. present (6)–(4)	Diff.	A.N. absent (2)–(3)	A.N. present (5)–(6)	Diff.	Net molar O_2/pyruvate ratio
1	3·39	13·21	9·82	3·52	7·50	3·98	2·47
2	1·54	12·00	10·46	3·28	7·42	4·14	2·52
3	5·59	11·04	5·45	3·98	6·30	2·32	2·35
						Av.	2·45

From Table 6 emerges the important result that the O_2/pyruvate ratio for phosphate-fumarate-adenine nucleotide catalysis of pyruvate oxidation averages 2·45; complete oxidation of pyruvate requires 2·50.

DISCUSSION

Two significant facts have emerged from the work described in the experimental part of this paper. First, it has been found that fumarate catalytically stimulates the O_2 uptake of dialyzed pigeon brain suspensions respiring in pyruvate only when adenine nucleotide is also present. Since it has already been shown (Long, 1943) that the stimulation of pyruvate oxidation by adenine nucleotide only occurs in the presence of inorganic phosphate, it follows that the combined activation must be regarded as phosphate-fumarate-adenine nucleotide catalysis. The other important observation is that fumarate is without effect on the utilization of pyruvate by this enzyme system and that its action consists solely in bringing about *complete* oxidation of that amount of pyruvate which was only *partially* oxidized in its absence.

In view of the finding that the rate of utilization of pyruvate by respiring suspensions of pigeon brain

is not increased by fumarate, the effect of the latter on pyruvate oxidation in tissues other than brain must now be considered. It has been pointed out earlier that most workers have investigated this problem using a medium containing malonate, so that their findings cannot be examined here. The few observations recorded in the literature, made in the absence of malonate, on tissues respiring in pyruvate which showed a marked increase in O_2 uptake when fumarate was added, are listed in Table 7.

of phosphate and adenine nucleotide only, the O_2/pyruvate ratio in pigeon brain averaged 1·20; when fumarate is also present in the medium the data in the present work indicate an O_2/pyruvate ratio of 2·50, i.e. complete oxidation. Thus the catalytic action of fumarate cannot be exerted directly on pyruvate itself since there is no change in the rate of pyruvate utilization. The conclusion to be drawn, therefore, is that fumarate is acting upon some intermediary oxidation product of pyruvate. In previous work (Long, 1943) evidence based

Table 7. *The effect of fumarate on pyruvate disappearance in animal tissues*

Tissue	No. of exp.	Fumarate conc. (M)	Pyruvate disappearance (mg./g./hr.) Fumarate absent	Fumarate present	% change due to fumarate	Reference
Rabbit liver	4	0·0083	5·64	4·93	−13	Annau *et al.* (1936)
	2	0·0058	4·35	3·50	−20	
Pigeon breast muscle	1	0·0025	8·19	9·89	+21	Krebs & Eggleston (1940)
	1	0·0025	12·80	10·91	−15	
Pigeon brain	6	0·0050	4·59	3·82	−17	Present work

The results of Banga *et al.* (1939) were not presented in a form suitable for inclusion in the above table.

It will be observed from Table 7 that the data of Annau *et al.* (1936) show a consistently smaller pyruvate disappearance in the presence of fumarate than in its absence. These results for rabbit liver are in complete agreement with those obtained in the present work on pigeon brain. The two results of Krebs & Eggleston (1940) do not allow any conclusion to be reached since they are at variance with one another. The only positive evidence in favour of an increased pyruvate utilization in the presence of fumarate is to be found in the work of Banga *et al.* (1939); their data for pigeon brain, however, are offset by the results obtained in the present work.

Taken as a whole, there seems to be no clear-cut evidence in the literature for an increased pyruvate utilization accompanying fumarate catalysis of the respiration rate in any animal tissue. On the other hand, the results of Annau *et al.* (1936) on rabbit liver and those given in the present paper on pigeon brain constitute good evidence for a slightly decreased pyruvate disappearance under the conditions of fumarate catalysis. In pigeon brain, this decreased rate of disappearance of pyruvate is due to the simultaneous oxidation of fumarate to pyruvate and it seems likely that the same explanation will hold for rabbit liver. When this simultaneous reaction is taken into account there is strong evidence for the absence of any effect of fumarate on pyruvate utilization.

It was shown (Long, 1943) that, in the presence

on data obtained for the R.Q. of pyruvate oxidation indicated that 60% of the carbon of pyruvic acid was oxidized to CO_2 in the presence of phosphate and adenine nucleotide only, the remaining 40% of the carbon being converted into unknown fixed acids. It seems very probable that fumarate is acting upon these fixed acids converting them to CO_2 and water.

SUMMARY

1. Fumarate stimulates the O_2 uptake of dialyzed pigeon brain suspensions, respiring in pyruvate, only when phosphate and adenine nucleotide are also present in the medium.

2. In the presence of fumarate there is a slightly decreased rate of disappearance of pyruvate under all conditions. This is explained by the fact that fumarate is simultaneously oxidized to pyruvate, and since the amount formed in this way almost exactly balances the decreased pyruvate disappearance, it is concluded that fumarate is without effect of the rate of utilization of pyruvate.

3. Under the conditions of phosphate-fumarate-adenine nucleotide catalysis, the O_2/pyruvate ratio was found to approach 2·50, i.e. pyruvate was oxidized completely to CO_2 and water.

4. It has been concluded from the data obtained that fumarate does not act directly on pyruvate, but on some intermediary product arising during pyruvate oxidation.

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The Glucuronidase Content of Animal Tissues and the Role of the Spleen in the Metabolism of Conjugated Glucuronic Acids

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It has been recognized for many years that the formation of conjugated glucuronic acids is an important result of the introduction into the body of many foreign organic compounds containing a hydroxyl or carboxyl group. That the main site of formation of these glucuronides is the liver has been established by Hemingway, Pryde & Williams (1934) using perfusion experiments and by Lipschitz & Bueding (1939) using the tissue slice technique.

The enzyme β-glucuronidase, which hydrolyzes glucuronides is found in several tissues (Masamune, 1934; Oshima, 1934, 1936; Fishman, 1938, 1939, 1940). Fishman has concluded, therefore, that the liver is not the sole site of glucuronide formation in the body. Fishman found the spleen to be a rich source of glucuronidase, the liver, ovary, uterus and kidney being relatively good sources. On feeding dogs or mice with menthol or borneol for a few days, Fishman (1940) observed that the glucuronidase content of liver, kidney and spleen increased, whereas that in the uterus remained the same and that in the ovary decreased. When oestrogenic substances (oestrone, oestradiol, etc.) were fed, the uterine glucuronidase increased but the liver glucuronidase remained unchanged (Fishman & Fishman, 1944). They also found that ovariectomy caused a decrease in uterine glucuronidase but not in the liver enzyme. Fishman (1940) concludes on the basis of his experiments that glucuronides are formed in organs other than the liver and that the uterus is the sole site of formation of the oestrogen glucuronides.

Lipschitz & Bueding (1939), on the other hand, found that liver slices were the only tissues to form significant amounts of glucuronides in the presence of menthol and borneol, the spleen being inactive and the kidney only slightly active in this respect. The question arises, therefore, whether the glucuronidase present in tissues is concerned with the synthesis of glucuronides (Florkin, Crismer, Duchateau-Bosson & Houet (1942), found glucuronidase to synthesize bornylglucuronide *in vitro* to a small extent from borneol and glucuronic acid), or whether it is concerned only with their hydrolysis. In this connexion, it should be mentioned that glucuronides when fed to animals are hydrolyzed in the body, for Quick (1928) has shown that on feeding benzoylglucuronide, about 50% of its benzoic acid is excreted conjugated with glycine in the dog.

That liver and spleen glucuronidase may be different is indicated by the work of Talbot, Ryan & Wolfe (1943) who found that the spleen enzyme prepared according to Fishman (1938) would not hydrolyze pregandiolglucuronide in urine satisfactorily, whereas a preparation from liver would.

The work of Mitsuba (1927) suggests that the spleen may play a part in the destruction of glucuronides. This worker found that, after splenectomy, the amount of glucuronic acid excreted by rabbits and dogs in response to anthranilic acid was greater than in the intact animal. In view of the fact that Mitsuba's method for estimating glucuronic acid is now considered unreliable and consequently that his experimental findings are inconclusive, it was decided to reinvestigate this problem. At the same time, more detailed figures than were hitherto available for the glucuronidase content of rabbit and rat organs are presented.

METHODS

Enzyme extracts for assay. The animals were killed by a blow on the head and the organs dissected out, minced finely with scissors, ground with silver sand and 3 vol. of water, shaken vigorously for 1 hr. and allowed to stand

overnight at room temperature. The extract was then centrifuged, the solution precipitated with 2 vol. acetone and the precipitate obtained shaken vigorously for 1 hr. with an amount of water equivalent to twice the weight of the original organ. The precipitate was removed on the centrifuge and the solution assayed immediately for glucuronidase.

Glucuronidase assay. One ml. of a suitable dilution of the enzyme extract (1/2 or 1/4) was incubated for 18 hr. at 37° with 2 ml. 0·1 M-acetate buffer, pH 5·2, and 1 ml. 0·025 M-Na *l*-menthylglucuronide solution (adjusted to pH 5·2 with acetic acid). Trichloroacetic acid (4 ml. of 10% (w/v)) was then added and the precipitate removed by centrifuging, the resulting solution poured into 6 × 1 in. pyrex tubes and the free glucuronic acid determined by the method of Miller & Van Slyke (1936) as modified by Fishman (1938), using 0·1% xylene cyanol F.F. (British Drug Houses Ltd.) as indicator in the titration. 1 mg. glucuronic acid liberated under these standard conditions = 1 unit of activity.

l-Menthylglucuronide was prepared according to Quick (1924). The Na *l*-menthylglucuronide was prepared by dissolving the free acid in the requisite amount of 0·1 N-NaOH, adjusting the pH to 5·2 and diluting to a standard volume.

Estimation of glucuronic acid in urine

(i) *Total glucuronides.* The method of Hanson, Mills & Williams (1944) was used. In this method it was found essential, for consistently accurate results, to observe the following four conditions:

(*a*) the quality of commercial naphthoresorcinol is variable and a fresh calibration curve must be made for each sample; (*b*) 0·25% naphthoresorcinol solutions are not reliable after 3 days even when stored in the dark at 0°; (*c*) it is essential to get complete separation of the aqueous and amyl alcohol layers in order to eliminate minute droplets of water in the final amyl alcohol extract, since the presence of water causes variable readings in the 'Spekker' photoelectric absorptiometer; (*d*) the water-bath must be boiling vigorously during the heating period otherwise variable results are obtained.

(ii) *Ether soluble glucuronides.* 2 ml. filtered urine + 2 ml. 2% (w/v) H_2SO_4 were extracted with ether for 1 hr. in a small continuous extractor. The ether extract was taken to dryness, the residue dissolved in hot water, made up to a standard volume and the glucuronic acid estimated as above. By this method 97–100% of *l*-menthylglucuronide added to urine could be recovered. The ether-soluble glucuronide in normal rabbit urine is of the order of 0–50 mg./day, but depends to some extent upon the rabbit.

Animal experiments. Adult rabbits were maintained on a constant diet of 200 g. cabbage and 50 g. crushed rat cubes per day, and the 24 hr. urine samples were collected each morning. After collecting the urine on the second day each animal was given by stomach tube a dose of 1 g./kg. *l*-menthol (British Drug Houses Ltd. $[\alpha]_D^{15°} = -50·7°$ in absolute ethanol) suspended in water and the urine collected for the following 3 days. All the extra glucuronic acid excreted in response to menthol is eliminated in 36–48 hr. For glucuronic acid estimations the urine was diluted 1/50 on the control days and for the 2 days after feeding menthol, 1/250. If D_1, D_2, etc., are the excretions of glucuronic acid on days 1, 2, etc., respectively, the extra glucuronic acid in response to menthol = $D_3 + D_4 - (D_1 + D_2 + D_5) \times \frac{2}{3}$.

RESULTS

Glucuronidase in rabbit and rat organs. The glucuronidase content of rabbit and rat liver, kidney and spleen are recorded in Tables 1 and 2.

Table 1. *Glucuronidase content of rabbit organs*

(Averages of seven animals—range in brackets.)

Tissue	Glucuronidase units*/g. fresh tissue	Organ (% of body wt.)	Glucuronidase units/100 g. body wt.
Liver	10·8 (7·6–14·5)	3·61	39·0 (25·0–46·6)
Kidney	1·8 (1·4–2·5)	0·72	1·15 (0·70–1·52)
Spleen	13·7 (11·4–17·2)	0·044	0·60 (0·52–0·64)

* One unit of glucuronidase activity = 1 mg. glucuronic acid liberated in 18 hr. at 37°, from 0·006 M-*l*-menthylglucuronide at pH 5·2 in 0·05 N-acetate buffer.

Table 2. *Glucuronidase content of rat organs*

(Averages of eighteen animals—range in brackets.)

Tissue	Glucuronidase units*/g. fresh tissue	Organ (% of body wt.)	Glucuronidase units/100 g. body wt.
Liver	5·65 (4·0–6·7)	3·81	21·5 (17·0–25·4)
Kidney	8·5 (5·5–11·9)	0·73	6·2 (4·4–8·5)
Spleen	29·1 (23·9–34·7)	0·33	9·7 (8·3–11·3)

* See footnote to Table 1.

Table 3. *Control excretion of glucuronides and percentage conjugation of* l*-menthol in normal rabbits*

Group	Rabbit no.	Control excretion of total glucuronic acid (mg./day)		Extra glucuronic acid excretion (calculated as % of *l*-menthol fed) Ether-soluble		Total		Difference (total ether-soluble)
1	22	254		48·5 : 46·0		68·0 : 65·3		
	23	238	Av. 252	36·2 : 54·9	Av. 45·3	59·0 : 70·0	Av. 63·4	18·1%
	28	266		48·0 : 43·0		56·0 : 70·0		
	29	252		39·0 : 46·8		58·0 : 60·9		
2	14	238		71·0 : 69·0		80·5 : 72·0		
	15	124		40·0 : 60·0		54·0 : 65·0		
				35·5		42·0		
	32	235	Av. 212	63·0 : 68·0	Av. 58·6	76·0 : 73·0	Av. 67·6	9·0%
				53·8		60·9		
	33	250		71·0 : 55·2		79·0 : 74·0		
		Average		52·7		65·6		12·9%

Effect of splenectomy on glucuronide excretion in rabbits. The total and ether-soluble glucuronides were estimated in the urine of normal rabbits before and after feeding *l*-menthol at a level of 1 g./kg. body weight, and the extra glucuronic acid excreted in response to the *l*-menthol, was calculated as a percentage of the menthol fed. The results obtained are shown in Table 3. The rabbits were splenectomized and divided into two groups. The first group was tested over a period of two weeks—starting a few days after splenectomy. The second group was rested for three weeks after splenectomy and then tested over a period of two weeks. The results obtained are shown in Table 4. For the sake of comparison, the results recorded in Tables 3 and 4 may be condensed (Table 5), when it will be seen that over the first and second week after splenectomy there is an increased conjugation of menthol, and that by the fourth and fifth weeks the level of conjugation has returned to normal.

Table 4. *Excretion of conjugated glucuronides in the rabbit after splenectomy*

Group	Rabbit no.	Time between splenectomy and menthol feeding (days)	Control excretion of total glucuronic acid (mg./day)	Extra glucuronic acid excretion (calculated as % of *l*-menthol fed)				Difference
				Ether-soluble (%)		Total (%)		
1	23	6	352	65·0		72·5		
	28	6	375 — Av. 335	64·5 — Av. 64·1		75·6 — Av. 74·1		10·0
	29	6	338	63·0		74·4		
1	23	13	239	69·0		87·0		
	28	13	282 — Av. 282	70·0 — Av. 63·0		89·0 — Av. 85·0		22·0
	29	13	324	50·0		80·0		
2	14	28	193	54		62		
	15	28	113 — Av. 190	35 — Av. 45		41 — Av. 53		8·0
	32	27	189	56		60		
	33	27	250	34		48		
2	14	35	200	65		72		
	15	35	136 — Av. 192	49 — Av. 63		65 — Av. 69		6·0
	32	34	204	57		70		
	33	34	228	60		70		

Table 5. *Extra glucuronic acid excreted in response to* l*-menthol in the rabbit. (Calculated as percentage of menthol fed)*

	Intact animal (%)	Av. 1st and 2nd weeks after splenectomy (%)	Av. 4th and 5th weeks after splenectomy (%)
Ether-soluble glucuronides	52·7	63·6	51·0
Total glucuronides	65·7	79·7	61·0
Difference	13·0	16·1	10·0

DISCUSSION

The figures given by Fishman (1940) for the glucuronidase content of animal (dog and mouse) organs are misleading since no account is taken of the size of the organ. When such figures are calculated on the basis of a standard animal weight a different picture is obtained. In the rabbit (Table 1) it will be seen that about 95 % of the total glucuronidase of this organism is contained in the liver and only about 1·5 % in the spleen. In the rat, however (Table 2), these figures are 60 and 25 % respectively. Organs other than the liver, spleen and kidney contribute only about 2 % to the total in the rabbit and about 5 % in the rat. The importance of spleen glucuronidase may, therefore, vary considerably from species to species. It is interesting to note in this connexion that Cannon & McClelland (1929) have shown that there is a species difference in splenic activity during an infection. According to the interpretation of Oshima (1936) and Fishman (1940) one would expect that the removal of the spleen would be followed by a decrease in the conjugation of menthol with glucuronic acid and since, in the rabbit, the spleen glucuronidase is very small compared with the liver glucuronidase, any alteration in amount of conjugation should be highly significant.

The figures recorded in Tables 3 and 4 show that in the two weeks following splenectomy there is first an increase in the normal output of urinary glucuronic acid and secondly an increase in the percentage conjugation of menthol. Both the increases in normal output of glucuronic acid and the percentage conjugation of menthol have declined again by the fourth and fifth week after splenectomy to the levels before splenectomy. These results are consistent with the view that the spleen plays some part in the breakdown of glucuronides in the body rather than with the view that the glucuronidase in all organs has a synthetic function. It is well known that after splenectomy certain functions of the spleen are taken on by other organs; this may also

apply to the destructive action of the spleen on glucuronides.

The figures recorded in Tables 3 and 4 bring out the interesting point that some of the glucuronide excreted in response to menthol is insoluble in ether. Since *l*-menthylglucuronide is easily soluble in ether, this may mean that a small proportion of the menthol may be transformed into a compound forming an ether-insoluble glucuronide. Possible transformations are either a dehydrogenation to a benzenoid compound containing a phenolic hydroxyl group, it being known that phenolic glucuronides are insoluble or only slightly soluble in ether, or an oxidation of either side chain with the formation of an aliphatic hydroxyl or carboxyl group. These types of compound also yield glucuronides which may be insoluble or only poorly soluble in ether.

SUMMARY

1. Figures are presented for the glucuronidase content of certain organs of the rabbit and the rat.

2. In the rabbit the normal output of glucuronic acid and the percentage conjugation of *l*-menthol with glucuronic acid are both increased for the first two weeks after splenectomy and then fall back to the level for the intact animal by the fourth week. The function of spleen glucuronidase is discussed in the light of these results.

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The Uptake of Oxygen by Blood *in vitro*

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In some recent experiments (Ramsay, 1944*b*) it was observed that the oxygen capacity of fresh blood often appeared lower than the value obtained from the same blood after a treatment involving reduction with titanous citrate or tartrate. This confirmed similar results obtained by Ammundsen (1941), who used carbon monoxide instead of oxygen and treated the blood with sodium hyposulphite ($Na_2S_2O_4$) instead of a titanous salt. The present author, however, also observed that if the blood was allowed to stand in air, the apparent oxygen capacity increased until it approached the 'total' value obtained initially after reduction. This communication reports a somewhat fuller investigation of the phenomenon, which resembles that encountered by Kallner (1945), who found that the carbon monoxide capacity also increased during exposure of blood to the air, but that the initial 'low' value was not observed at all if the pressure of carbon monoxide used for the saturation was 150 mm. instead of the 25 mm. originally recommended by Van Slyke & Hiller (1928).

METHODS

Specimens. Since blood from horses gave the most striking results in the earlier investigation, attention was concentrated on that species, but observations were made on other animals, principally sheep, cattle and pigs, and also man. In most of the animals blood was drawn from the external jugular vein into flasks containing oxalate, and suitable precautions were taken to prevent the access of air until the experiments were begun. Except in the case of the pig bloods, this was usually within 5–10 min., although occasionally the delay was reduced to 3 min. Tests showed, however, that a delay of up to at least 3 hr. did not seem to affect the general trend of the results.

Apparatus for aeration (or exposure to gas mixtures). The usual method of exposing blood to air in a mechanically rotated tonometer is not suitable for use with horse blood, which may show a sedimentation rate in Westergren tubes of over 3 mm./min. Moreover, even with bloods having a normal slow sedimentation rate this method of oxygenation is subject to small but significant errors (Roughton, Darling & Root, 1944). In the present work, therefore, treatment of blood with air or other gas mixtures was done in a vessel in which the blood was less subject to sedimentation and related errors, and which was easier to manipulate than a tonometer. The vessel, which is similar to, but simpler than that employed by Brooks (1935), is illustrated in Fig. 1. The large aperture is closed with a one-holed

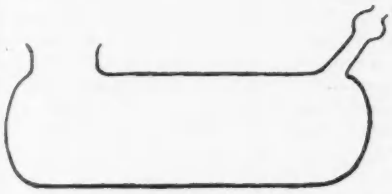

Fig. 1. Vessel for the aeration of blood samples. Overall length about 10–11 cm.

stopper carrying a short glass tube of 1 mm. bore. This serves as an exit for the gas, saturated with water vapour at the appropriate temperature (all experiments were done at room temperature), which is passed in through the small side-tube at a rate of 5–10 l./hr. The vessel is shaken mechanically as rapidly as it is possible without causing serious foaming of the contents. It is unnecessary to interrupt the stream of gas during the removal of samples, which can be done within a few seconds of the cessation of shaking by withdrawing the capillary glass tube and inserting the tip of a pipette. As a means of equilibrating blood with air or any other gas mixture this method appears less efficient than the rotating tonometer, because the latter presents a larger and continually changing surface of blood to the atmosphere. This deficiency, even if it be real, is not material to the present experiments.

Blood-oxygen determinations. The usual manometric Van Slyke technique was used, saponin in the ferricyanide reagent being replaced by 0·15–0·20% oleyl sodium sulphate. Too great an excess of this substance not only causes stopcocks to leak, but also impairs greatly its haemolytic action. The correction for dissolved oxygen was that recommended by Sendroy, Dillon & Van Slyke (1934).

Total haemoglobin. This was determined by the method of Ramsay (1944*b*). To avoid low results, the Van Slyke apparatus was washed as a routine before each analysis, once with 1% (v/v) nitric acid and then once with 2·5% (w/v) $Na_2HPO_4.12H_2O$. Probably the nitric acid inactivates by oxidation traces of sulphur compounds retained by the mercury from the $Na_2S_2O_4$ used in the previous analysis. The sodium phosphate washes out the residual nitric acid and leaves the apparatus at a faintly alkaline reaction, which is least harmful to haemoglobin during the early stages of the analysis.

Iron. This was determined by the method of Ramsay (1944*a*), with one minor alteration. It has been found that overheating in the digestion process leads to very low results. The error cannot be due to loss of iron, since it is decreased or eliminated by prolonged boiling of the digest after dilution, followed by an extremely slow titration. Neither can the error be due to phosphate, since low results engendered by the presence of relatively large quantities of that ion are not corrected by boiling. Since it has also been found that the presence of traces of $HClO_4$ does not falsify the titration, and thus that the removal of the last trace of this acid is not vital, overheating of the digest is now strictly avoided by terminating the digestion immediately on the disappearance of the yellow colour described before. The digest is boiled for a few seconds after dilution, and is then cooled to room temperature before the titration is done.

RESULTS

Preliminary investigations (Ramsay, 1944*b*), which now seem perhaps to have been inadequate, suggested that 15 min. aeration should suffice for the saturation of blood with oxygen. In that work, the time of aeration was therefore 15 min., but after it was observed that the oxygen capacity, particularly of horse blood, appeared to increase when the blood was allowed to stand exposed to the atmosphere, it became of interest to determine the effect of a more vigorous exposure of blood to the air. Samples of horse blood fresh from the animal were shaken with air for periods of time far in excess of the 15 min., and the apparent oxygen capacity was determined at intervals. The results were variable, but seemed to fall broadly into three groups, although no sharp differentiation could be made between one group and the next. The groups comprised respectively those bloods in which oxygenation was rapid and complete, those in which it was rapid but not quite complete, and those in which it was very slow. The completeness of oxygenation was judged by comparing the apparent oxygen capacity with the experimentally determined total haemoglobin (as vol. O_2%). One example from each of these groups is illustrated graphically in Fig. 2, where the oxygen taken up at any given time is expressed as a percentage of the total possible. Oxygenation was never complete in 5 min. This observation is important in connexion with later experiments. Occasionally, aeration for 20–30 min. resulted in 100% oxygenation, but it was more usual for oxygen to be taken up more or less rapidly until 96–98% saturation was reached, after which the curve flattened out, either partially or altogether. In a few cases it proved necessary to aerate the blood for upwards of an hour before oxygenation approached completion. These experiments have been repeated with blood specimens from man, sheep, and a few cows and pigs. Minor differences among the species were noticed. Sheep and cattle bloods took up oxygen more rapidly than human bloods, and the majority of these seemed

more rapid than the horse bloods. It is difficult to fit in the pig bloods, since they were unfortunately all 2 or 3 days old (they were kindly posted to me by Prof. E. J. King, Hammersmith), but a few of

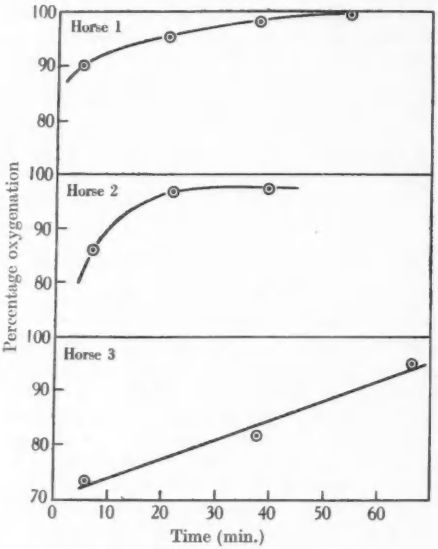

Fig. 2. Rate of uptake of oxygen by three different horse bloods.

them required an exceptionally long aeration period for oxygenation. The most marked and characteristic species difference noticed may be summarized by stating that all sheep and cattle bloods examined were at least 99% oxygenated after 30 min. aeration, whereas figures as low as 96–97% were not uncommon with human and horse bloods, and there were rare cases in which the deficiencies were considerably greater. The analytical error is low enough for these differences to be considered highly significant.

It was claimed by Conant, Scott & Douglass (1928) and confirmed by the author (Ramsay, 1944*b*) that after evacuation and treatment with titanous tartrate or titanous citrate, blood might be completely oxygenated by aeration for as short a period as 5 min. That oxygenation is indeed complete in such cases is shown by the fact (see Table 2) that each milligram-atom of iron in the experimental blood samples combines, under such treatment, with almost exactly 22·40 ml. of oxygen. This observation stands in marked contrast to those described in the preceding paragraph. The explanation offered by Kallner (1945) for some apparently similar phenomena is that fresh blood contains carbon dioxide, which exerts some inhibitory effect on the uptake of carbon monoxide (or presumably oxygen, although Kallner did not discuss this gas). Exposure to air removes carbon dioxide, with the result that blood treated in this way should take up carbon monoxide more completely, as is of course the experimental observation. No evidence was given in support of this theory, and the following series of experiments was designed not only to illustrate the striking change in properties which results from previous aeration of the blood, but also to test whether Kallner's hypothesis would serve as an explanation of the behaviour of venous blood *in vitro* towards atmospheric oxygen.

A fresh blood sample was divided into two portions. Portion *X* was exposed to a vacuum for a time shown by experiment to be sufficient for the removal of practically all oxygen and carbon dioxide. It was then aerated for 5 min., and its apparent oxygen capacity determined (see Table 1, line (i)). In the meantime, portion *Y* was aerated direct, and its apparent oxygen capacity determined after 5 min. (line (ii)) and 60 min. (line (iii)). Portion *Y* was then deoxygenated and decarbonated *in vacuo*, and re-aerated for 5 min., after which the apparent oxygen capacity was again determined (line (iv)). Total haemoglobin and iron were also determined, and expressed as vols. of O_2%, the assumption being made in the case of iron that 55·84 mg. of iron combine with 22·40 ml. of oxygen. These experiments were done on the blood of horses and of sheep, since these two species provided the extremes of behaviour when blood samples were simply shaken in air. The results of two representative experiments on each species are given in Table 1.

Table 1. *Oxygen uptake of blood after various treatments*

Treatment of blood	Apparent oxygen capacity (vol. of O_2%) Horse A	Horse B	Sheep A	Sheep B
(i) Evacuation and 5 min. aeration (portion *X*)	13·09	13·60	11·69	11·57
(ii) 5 min. aeration (portion *Y*)	13·91	13·57	16·11	13·26
(iii) 60 min. aeration (portion *Y*)	15·89	15·01	18·18	13·43
(iv) 60 min. aeration, evacuation, and 5 min. aeration (portion *Y*)	16·03	15·31	16·64	13·41
(v) Reduction with Ti (total oxygen)	16·15	15·39	18·20	13·52
Calculation from Fe (55·84 mg. Fe = 22·40 ml. O_2)	16·38	15·29	18·40	13·68

On examination of Table 1 it is evident that 60 min. aeration, as might be expected, gives a close approximation to the actual total possible oxygen capacity (compare line (iii) with lines (v) and (vi)). Some horses provided exceptions to this rule, the discrepancies in such cases ranging up to about 4 %. As is shown by line (ii), 5 min. aeration of fresh blood is quite insufficient for thorough oxygenation. Moreover, and this is important from the point of view of Kallner's hypothesis, evacuation of fresh blood, followed by 5 min. aeration, still does not lead to complete oxygenation (see line (i)). On the other hand, if the blood is thoroughly aerated and then evacuated before 5 min. aeration, oxygenation is usually complete (compare line (iv) with line (iii) and line (v)). One or two sheep bloods behaved exceptionally during these re-aeration experiments, in that they were not completely oxygenated in 5 min., although the oxygen taken up was always more than when the fresh evacuated blood was aerated for the same period. No explanation can be offered, but it was noticed in these cases that the early stages of oxygenation appeared to be sluggish; that is to say, the change in colour of the blood from blackish to bright red was unusually long delayed.

DISCUSSION

When it is borne in mind that the total haemoglobin determination is not affected by previous exposure to air, and that this determination requires only 5 min. aeration, it is interesting to note that treatment of the blood with a reducing agent such as titanous citrate or tartrate increases the rate of uptake of oxygen, under the conditions of these experiments, to much the same extent as exposure of the blood to air for an hour. The experiments of Kallner (1945) suggest the possibility that the same may be true of the treatment with $Na_2S_2O_4$ in the carbon monoxide method for total haemoglobin.

An attempt was made to confirm these experimental results by substituting treatment with oxygen-free nitrogen for exposure to a vacuum, but it was unfortunately found (cf. Mathison, 1911) impossible to remove either oxygen or carbon dioxide completely from blood by this process alone.

It may be noted in passing that the rate of uptake of oxygen from air containing 5 % carbon dioxide was not greatly different from the rate when ordinary laboratory air was used. Such slight differences as were sometimes observed may have been due to alterations in acidity. This is slight circumstantial evidence against the hypothesis of Kallner, but a more decisive refutation of the suggestion that his hypothesis might apply to these oxygen experiments may be gleaned from Table 1. Evacuation, which was shown experimentally to remove practically all carbon dioxide, only increases the rate of uptake of oxygen if it is preceded by thorough oxygenation. The presence of carbon dioxide can therefore not be regarded as a decisive factor.

One possible explanation of the results in Table 1 is that oxygen and the reducing agents may alter the permeability of the erythrocyte to oxygen. Although this seemed unlikely, because the work of Hartridge & Roughton (1927) suggests that the time lag imposed by the cell membrane is of the order of 0·01–0·1 sec., some experiments were done to test this point. It was thought that a comparatively gross experiment would serve to detect a gross effect, so the rate of uptake of oxygen by fresh blood was measured in both the presence and the absence of the least quantity of cetyl trimethyl ammonium bromide required to bring about complete haemolysis in a few seconds. The results, plotted graphically for two typical bloods in Fig. 3, are again given as percentages of the total possible oxygen uptake.

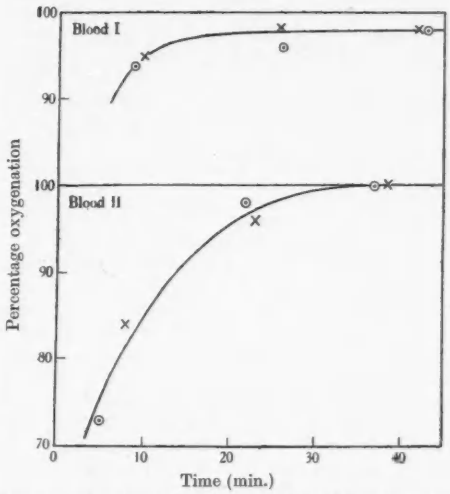

Fig. 3. Rate of uptake of oxygen by two specimens of blood, measured in both the presence and the absence of cetyl trimethyl ammonium bromide. ⊙ Points for unhaemolyzed blood. × Points for haemolyzed blood.

It is evident from Fig. 3 that haemolysis by cetyl trimethyl ammonium bromide does not increase the rate of oxygen uptake as measured in these experiments. Hence, since complete removal of the cell membrane is without effect, it seems unlikely that permeability of that structure can be a factor of importance. It should be stated that the method of expressing the results was chosen in view of the confusing fact that in the haemolyzed blood the total haemoglobin was always 2–5 % lower than in the untreated blood. This was doubtless caused by the denaturing action of the haemolytic detergent.

The belief is now fairly general that normal blood may contain ferrihaemoglobin in amounts too small to be detected by ordinary spectroscopic methods, such as that of Wendel (1938). Even if gasometric evidence be laid aside, there is still support for this belief in the spectroscopic measurements of Havemann, Jung & Issekutz (1939) and the more recent work of Paul & Kemp (1944). Examination of Table 1 suggests that the results summarized there would be consistent with the hypothesis that fresh venous blood contained larger amounts of ferrihaemoglobin than have hitherto been suspected, and that this disappeared fairly rapidly when the blood was exposed to the atmosphere. Such disappearance is known to take place in cases of experimental and pathological ferrihaemoglobinaemia (Campbell & Morgan, 1939; Cox & Wendel, 1942). This theory is not easy to test. Direct spectroscopic observations may not be very reliable, but efforts have been made to obtain some indirect evidence. Unpublished experiments in this laboratory have shown that two molar proportions of KCN inhibit practically completely the reduction of ferrihaemoglobin by even a ten-fold excess of ascorbic acid. Consequently, it was thought worth while to try the effect of KCN on the rate of oxygenation of fresh venous horse blood. No effect whatever could be detected, whereas it would be reasonable to suppose that a high concentration of ferrihaemoglobin in the blood would have led to a slowing of the rate of oxygenation in the presence of KCN, which might have retarded the rate of disappearance of ferrihaemoglobin. For similar reasons it was thought possible to test the hypothesis by observing the effect of sodium or ethyl iodoacetate, since these substances, by reacting with sulphydryl groups, might also decrease the rate of reduction of ferrihaemoglobin. Neither of these substances, however, had any effect whatever on the rate of oxygenation. These experiments are unfortunately subject to criticism on the ground that the red cell may not be sufficiently permeable to either cyanide or iodoacetate for any effect to take place in the short time available. Even on theoretical grounds, however, the disappearing ferrihaemoglobin theory is unlikely to be correct. If it were, then, since arterial blood is at least 95% saturated with oxygen (98·5%, according to the recent work of Drabkin & Schmidt, 1945), it would be necessary to assume that the circulating corpuscle accumulated a considerable proportion of ferrihaemoglobin which reverted more or less completely to ferrohaemoglobin each time the corpuscle passed through the lungs. Since the possibility of influence by carbon dioxide has been excluded by experiment, the only factor altering the proportion of ferrihaemoglobin would thus be the pressure of oxygen. Provided that the redox potential lies within certain limits, it is a simple matter to give a physico-chemical argument demonstrating the theoretical possibility of this (some related phenomena were discussed by Conant & Fieser, 1924), but the fact that a high proportion of the intracorpuscular glutathione appears to be in the reduced condition (Woodward & Fry, 1932; Prunty & Vass, 1943) makes it extremely unlikely that the redox equilibrium is at a level suitable for the operation of such a mechanism. In the absence of definite favourable evidence, then, it seems impossible to maintain the hypothesis that the behaviour of fresh venous blood on exposure to air is due to the disappearance of ferrihaemoglobin. It must be emphasized, however, that in the absence of detailed knowledge of the intracorpuscular redox equilibrium, this criticism need not necessarily apply to the small residual oxygen- and CO-capacity discrepancies noted by Ramsay (1944*b*) and by Ammundsen (1941). The few larger discrepancies noted by the author in samples of horse blood were probably due to incomplete oxygenation, undetected because of those variations in the rate of oxygen uptake which are the subject of the present discussion.

Since blood is normally almost completely saturated in its passage through the lungs, it seems unlikely that this work has any significance for the living animal. If current figures for the oxygen content of arterial and venous blood be accepted (and there seems to be no reason to doubt them), then most of the circulating haemoglobin must spend the greater part of its time at least partially oxygenated. Hence, since the experiments described here show that thorough aeration is necessary to prevent the reaction between haemoglobin and oxygen from becoming sluggish, conditions in the living body should tend to prevent sluggishness. Indeed, it seems difficult to avoid stating the possibility that the observed sluggishness may develop after the blood is drawn, while it is kept, even for a short time, in a partially oxygenated condition. One is reminded of the observation, put on a sound footing by Brooks (1935) that ferrohaemoglobin is much more rapidly oxidized to ferrihaemoglobin by oxygen at low pressures than at pressures sufficient to permit the existence of a large proportion of oxyhaemoglobin. The stabilizing effect of oxygen may be of general application.

There is a suggestion in the figures given in Table 1 that both the 'total' haemoglobin and the oxygen capacity after 60 min. aeration are in good agreement with the total iron of the blood. Since such agreement is a matter of some importance in connexion with the selection and adjustment of standard haemoglobin methods, it has been thought worth while to add Table 2, in which the experimental oxygen, both 'total' and after 60 min. aeration, calculated for 55·84 mg. experimental iron,

is given for each of ten consecutive bloods, six from horses and four from sheep. Details are given in order that the values may be compared for individual specimens. The theoretically perfect result is, of course, 22·40 ml. in every case. Some similar experiments were done on the equivalence of Van Slyke oxygen and iron in 25 human bloods by Jope, Jope & O'Brien (1943). The results of these workers, recalculated to the same basis, are included in the table for comparison.

Table 2. *Equivalence of experimentally determined oxygen and iron*

(Theoretical value 22·40 ml. O_2/55·84 mg. iron.)

Exp.	Van Slyke oxygen after 60 min. (ml. O_2/ 55·84 mg. Fe)	'Total' oxygen (ml. O_2/ 55·84 mg. Fe)	Jope *et al.* (1943)
1H*	22·71	22·76	—
2H	21·75	22·10	—
3H	22·00	22·55	—
4H	21·72	22·54	—
5H	22·03	22·00	—
6S	22·16	22·19	—
7H	21·99	22·18	—
8S	22·38	22·41	—
9S	22·13	22·20	—
10S	22·25	22·40	—
Mean	22·11	22·33	22·11 (25 results)
Difference of mean from theoretical	−1·3%	−0·3%	−1·3%
Range	21·72–22·71	22·0–22·76	20·49–23·34
S.D. as % of mean	1·3%	1%	3·3%
S.D. of Fe method	0·5%	0·5%	†
S.D. of O_2 method	0·5%	0·5%	†

* H = horse, S = sheep.
† Methods stated by the authors to be reproducible to within ±0·2–0·5%.

It appears from this table that the total oxygen capacity, measured after reduction, corresponds very closely indeed to the total iron of the blood. There are no discrepancies outside the limits of experimental error, and the mean, 22·33 ml. of O_2/55·84 mg. of Fe, indicates that virtually the whole of the blood iron is in combination as ferrohaemoglobin or some substance which combines with oxygen after treatment with a suitable reducing agent. Much of the so-called serum iron may be haemoglobinoid, at least in origin, since it is not uncommon to find plasmas or sera which, although not abnormal in colour, show oxyhaemoglobin bands when a sufficiently thick layer is examined spectroscopically. Moreover, it has recently been suggested that physiological intravascular haemolysis may occur during exercise (Gilligan, Altschule & Katersky, 1943). The Van Slyke oxygens after aeration for 60 min. also agree well with the iron, although there is a little more scatter, and the mean is lowered by one or two results which suggest either that even 60 min. aeration of these bloods was inadequate, or else that they did actually contain small amounts of ferrihaemoglobin. The summary of their results published by Jope *et al.* (1943) indicates that some of their values must have been 7% below their mean, or a full 8% below the theoretical value. It seems possible that in such cases oxygenation was incomplete.

That the apparatus used by the author for aeration is an efficient oxygenator is shown by the fact that after aeration and complete deoxygenation *in vacuo*, blood may be completely oxygenated in 5 min. It therefore seems justifiable to draw to the attention of analysts the fact that the rate of uptake of oxygen by blood is variable, and may on occasion be much slower than has been generally believed. Distinct species variations can be detected, but perhaps the most important practical point is that in all cases the rate is made more rapid by thorough oxygenation of the blood. This suggests that the converse may be true: that the keeping of blood in a low state of oxygenation may have an adverse effect on the rate at which it will subsequently take up oxygen. These difficulties may be avoided if the blood is treated with a suitable reducing agent, since oxygenation may then be carried out in 5 min. It is unfortunate that none of the possible explanations of these observations has so far proved satisfactory.

SUMMARY

1. A simple apparatus for the oxygenation of blood or its treatment with any gas mixture is described. Its advantages over the rotating tonometer are discussed.

2. When blood is aerated in this apparatus, a period of 15–60 min. is usually required for 98–100% oxygenation. The previous observation of frequent small deficiencies in oxygen capacity is confirmed.

3. If the blood has been freshly oxygenated, and then deoxygenated *in vacuo*, only 5 min. aeration is usually required for reoxygenation, as in the titanous citrate or tartrate method for total haemoglobin.

4. The following possible explanations are discussed: presence of carbon dioxide, presence of ferrihaemoglobin, alterations in the permeability of the erythrocyte to oxygen. It is found necessary to reject all of these.

5. Very good agreement is observed between total iron and total haemoglobin or oxygen capacity after 60 min. aeration.

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The Component Acids of Cow Colostrum Fat

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Though much work has been done on the composition of milk fat, little attention has so far been paid to the fat secreted during the first few days after parturition. Baldwin & Longenecker (1944*a*) studied the fatty acid distribution in the fat of human milk during the first few days of lactation and found that it was different from that of mature milk fat. The results of these workers indicated that as the period of lactation progressed from the first day's colostrum to mature milk, the amount of the acids of greater molecular weight than C_{18} decreased and the amount of C_{12} and C_{14} acids increased. Later, Baldwin & Longenecker (1944*b*) investigated the colostrum fat of a single cow collected during the first 4 days after parturition and reported that its fatty acid composition was very similar to that of a typical butter fat. The results obtained were only from samples collected from a cow of unspecified breed and comparisons were made with the results of the analysis of typical butter fat by Hilditch & Longenecker (1938). It is possible that if a sample of butter fat from the same cow had been collected and analyzed at a later period some differences might have been observed. Apart from these investigations no work appears to have been carried out on this subject. In the present study attempts were made to collect colostrum fat from some of the common breeds of cattle in India and the results obtained are now presented.

METHODS

The colostrum from which the fat was extracted for the present studies was collected from four different breeds of cows, namely, Sindhi, Gir, Tharparkar and Sahiwal. Milking was done twice a day. In the case of each animal material collected during each 24 hr. after parturition was mixed together, diluted with water and the cream separated. Butter fat was prepared direct from the cream by the 'boiling off' method. The butter fat prepared from each 24 hr. collection from every animal was first analyzed for its general chemical characteristics. The collection of butter fat was continued during the first 15 days of lactation and samples from the first 5 days and the tenth day of lactation were used in the present studies.

During the dry period the cows were receiving 3 lb. of a concentrate mixture (wheat bran, ground-nut cake, grain husk and grain in the ratio of 4:2·5:2·0:1·5) in addition to ½ lb. each of extra grain husk and ground-nut cake. Four weeks before calving pregnant animals were fed 3 lb. of the usual concentrate mixture mentioned above with 3 lb. of wheat bran. For the first 4 days after calving the animals received 8 lb. wheat bran, 1 lb. of *jaggery* (crude cane sugar), with a roughage consisting of 55 lb. green grass and 3 lb. ragi straw. From the fifth day of lactation onwards the animals were fed, together with the usual amount of roughage as mentioned above, different amounts of the common concentrate mixture according to milk yield.

Representative composite mixtures of the colostrum fat during the initial days of lactation were obtained by mixing equal quantities of the fat collected from the different cows

on the corresponding days of lactation. This mixed butter fat for each day of lactation was first analyzed for its physical and chemical characteristics and then subjected to detailed analysis by ester fractionation according to the method of Hilditch, as modified by Smith & Dastur (1938). The fat was converted into methyl esters directly and then the lower components fractionally separated from the whole bulk, the higher members being separated into liquid and solid acids by Twitchell's lead salt method, methylated and also fractionally distilled. The detailed results obtained for the composition of the three main fractions, the lower, solid and liquid esters of the colostrum fat on the first day are given by way of illustration in Tables 2 and 3, while the final figures are recorded in Table 4. Unfortunately the small amounts of material available for analysis prevented isolation of the lower unsaturated acids C_{10}, C_{12} and C_{14} in the present work. Their existence in butter fat has, however, been established beyond doubt by Bosworth & Brown (1933), Hilditch & Paul (1936) and other workers.

RESULTS

The chemical characteristics of the individual samples of colostrum fat and those of composite mixtures are shown in Tables 1 and 1*a*. Table 1 shows clearly that as time goes on there is a marked progressive change in the nature of the fat secreted by individual animals as judged by the butyro-refractometer reading, and the iodine, saponification and Reichert values. These changes were very similar for all the animals except cow 531, in which the transition was not so well marked. In every case the butyro-refractometer reading decreased from 45·4 to about 42·1 during the progress of lactation. The saponification value gradually increased from about 213 to 232. The Reichert value rapidly increased from about 13 on the first day to 20 on the second day and then gradually increased to about 30. The iodine value decreased from about 40 to 32. The results of analyses of the composite mixtures also reveal the same gradation in all their chemical constants (Table 1*a*). The most interesting observation concerns, perhaps, the physical state of the colostrum samples; the melting-point of the fat gradually decreases with the progress of lactation. This may be attributed to the presence of larger amounts of stearic acid in the colostrum fat relative to the normal butter fat collected later.

The decrease in the butyro-refractometer reading and iodine value, and an increase in the saponification and Reichert values of the fat secreted by the animal as the colostrum fat changed to normal butter fat suggested a marked increase in the amount of fatty acids of low molecular weight and a slight decrease in the content of unsaturated acids. This was confirmed by detailed analysis based on fractional distillation methods. Tables 2 and 3 giving the detailed analytical results for fat in the first day of lactation, illustrate the methods. Table 4, wherein the component fatty acids for all the samples by weight and molar percentages are presented in summarized form, shows clearly that there is a regular increase in the proportion of the low molecular weight fatty acids during the transition from the first day's colostrum fat to mature milk fat on the tenth day of lactation. The butyric acid content increased from 2·2 to 3·6 %. The amount of caproic acid in the fat was almost negligible on the first day. It gradually increased up to 1·5 % on the fourth day and then decreased to 0·6 %, at which level it remained constant. Caprylic, capric and lauric acids also increased till the fourth day after parturition and remained fairly steady. There appeared to be little change in the proportion of myristic acid, the slight increase in its content being counterbalanced by a slight decrease in stearic acid. The palmitic acid content of the secreted fat slightly increased on the second day after calving and then became constant. The amount of arachidic acid remained almost constant. The changes in the lower acids can be seen more clearly by reference to molar percentages. There is an increase of about 10 mol. % in the lower acids up to and including C_{14}. This is mainly accounted for by the fall of almost 6 mol. % in oleic acid. The fall in the percentage of stearic acid is also evident. In the unsaturated acids up to C_{14} there is virtually no change. The amount of hexadecenoic acid increased at first and then decreased to almost the original value. The chief change appeared to be in oleic acid, which decreased from 34·1 on the first day to 27·5 on the fourth day and then slightly increased to 29·3 on the tenth day of lactation. The amount of linoleic acid decreased gradually to almost one-quarter of its amount present in the first day's fat. There was no marked change in the C_{20-22} unsaturated acids. These changes, a decrease in the quantity of higher acid and an increase in the amount of lower acids, may well be associated with the functional development of the lactating mammary gland following parturition. A gradual decrease in the percentage of oleic acid and low amounts of volatile acids present in the colostrum fat are in close agreement with the findings that colostrum fat is richer in oleic acid and contains less volatile fatty acids than normal milk fat (Sommerfeld, 1909; Nilson, 1891). In comparison with the component fatty acids of human colostrum fat (Baldwin & Longenecker, 1944*a*) several differences in composition are apparent. The most interesting observation is the presence of larger amounts of low molecular weight fatty acids and smaller amounts of C_{20} and C_{22} acids in cow colostrum fat than in human colostrum fat. The first day's cow colostrum fat compares favourably with the mature human milk fat, the percentage of lower acids up to C_{14} being almost the same (16·4 and 17·8 respectively). The only point of

Table 1. *General analytical characteristics of colostrum and milk fats*

Day of lactation	Butyro-refractometer reading (at 40°)	Saponification value	Iodine value	Reichert value	Polenske value	Kirschner value
Cow 82. Gir (2nd lactation)						
1	45·2	213·7	38·60	13·16	0·95	12·31
2	44·2	220·4	35·20	18·99	1·40	17·31
3	43·0	224·2	33·40	22·22	1·61	20·75
4	42·1	224·8	29·70	23·39	2·20	21·62
5	41·6	226·2	30·00	29·21	2·30	25·86
10	41·0	228·2	29·45	32·82	1·27	30·54
15	41·0	229·3	33·95	30·71	1·99	29·17
Cow 251. Sindhi (11th lactation)						
1	45·3	215·4	38·30	14·75	1·10	12·02
2	44·0	223·0	35·30	21·32	1·25	19·87
3	42·9	224·4	32·80	24·35	1·40	22·80
4	41·3	226·0	31·00	30·23	1·25	26·81
5	42·0	231·2	30·60	31·30	2·10	28·26
10	42·5	230·1	34·20	31·40	2·10	27·80
15	42·0	231·4	32·10	31·77	1·75	27·14
Cow 257. Tharparkar (4th lactation)						
1	45·5	213·3	41·58	13·98	0·97	12·54
2	44·0	221·9	38·68	21·45	1·59	20·07
3	42·8	224·8	34·53	23·28	2·30	21·78
4	42·2	226·0	34·71	28·64	2·20	24·91
5	42·2	228·6	34·84	28·76	1·89	25·11
10	42·7	228·2	35·72	30·79	1·69	27·10
15	42·8	227·4	36·83	29·67	1·79	26·21
Cow 531. Ayrshire-Hariana (12th lactation)						
1	43·9	219·3	32·98	15·82	1·21	13·61
2	42·8	223·1	28·98	19·05	1·79	17·33
3	42·0	225·2	29·75	21·34	1·89	18·48
4	41·3	226·1	28·28	26·18	2·20	22·84
5	41·0	228·0	28·75	28·43	2·30	24·62
10	42·7	228·0	30·54	27·63	2·10	23·66
15	41·3	229·2	29·82	29·22	2·50	25·83
Cow 64. Sahiwal (3rd lactation)						
1	45·4	213·5	42·79	14·59	0·75	12·66
2	45·2	214·9	41·62	18·05	0·75	15·12
3	43·9	218·0	38·39	19·48	1·15	16·87
4	43·4	221·7	36·12	21·74	1·40	19·05
5	42·7	226·0	33·48	24·27	1·70	20·77
10	42·1	228·0	31·32	27·07	1·70	23·93
15	41·6	230·0	30·84	26·41	1·30	23·30
Cow 64. Gir (5th lactation)						
1	44·2	218·0	38·42	13·98	1·20	12·92
2	43·8	220·3	36·53	19·00	1·59	17·38
3	43·5	222·0	35·12	22·35	1·79	20·95
4	43·1	225·1	32·50	24·95	1·99	23·68
5	42·8	227·4	31·67	26·06	1·79	22·46
10	42·3	229·6	32·01	32·02	1·79	29·20
15	42·5	228·1	32·16	29·00	1·55	26·05

Table 1*a*. *Analytical characteristics of the composite mixtures of colostrum fat*

Day of lactation	Butyro-refractometer reading (at 40°)	Saponification value	Iodine value	Reichert value	Polenske value	Kirschner value	Melting-point
1	45·4	213·1	39·79	14·92	1·10	11·21	38·7
2	43·8	220·4	35·30	22·18	1·40	20·61	34·3
3	43·3	224·0	33·51	24·27	1·70	22·23	34·2
4	43·2	225·1	32·06	25·75	1·95	22·41	33·9
5	42·8	226·8	31·56	27·01	2·04	23·24	33·9
10	42·1	228·2	31·60	30·56	1·96	26·20	33·8
15	41·8	229·6	31·82	29·72	1·98	25·92	—

Table 2. *Fractionation of the methyl esters prepared from* 300 *g. of cow colostrum fat (first day of lactation)*

Fraction	Boiling-point at 2 mm. up to	% of total esters	Mol. wt.	Iodine value	Fraction	Boiling-point at 2 mm. up to	% of total esters	Mol. wt.	Iodine value
				Lower esters					
1	(Methyl butyrate)	2·62	—	—	7	127	0·60	211·9	6·42
2	54	0·34	156·7	3·08	8	137	0·97	229·6	7·18
3	80	0·59	163·6	3·93	9	140	1·02	239·2	7·56
4	92	0·50	181·4	5·46	10	145	1·13	244·0	7·52
5	103	0·61	190·8	7·87	11	150	0·86	247·9	8·04
6	115	0·42	200·9	7·08	12	153	1·03	255·2	8·78
						Total	10·69		
				Solid esters					
13	154	0·85	254·2	0·54	24	169	2·20	276·8	2·60
14	159	1·12	263·7	0·52	25	169	2·28	277·1	2·92
15	162	1·35	268·5	0·98	26	170	2·14	277·4	2·91
16	162	1·80	270·1	1·56	27	170	2·06	280·9	3·06
17	164	2·13	271·9	1·56	28	174	2·58	281·3	3·53
18	165	2·04	272·4	1·84	29	174	2·24	282·8	4·06
19	165	2·18	273·0	1·93	30	176	2·28	286·4	4·51
20	164	2·58	273·3	1·93	31	180	2·18	289·4	6·63
21	167	2·45	273·3	2·04	32	185	1·29	293·8	5·88
22	168	2·12	273·5	2·27	—	Residue	1·44	309·1	8·28
23	169	2·43	275·8	2·66		Total	41·74		
				Liquid esters					
33	115	0·36	238·2	19·28	44	172	2·00	289·8	69·48
34	142	0·71	244·5	19·62	45	172	2·42	291·2	71·06
35	144	1·02	246·8	19·54	46	173	2·64	292·7	76·92
36	149	1·36	250·7	19·58	47	174	2·50	293·5	80·72
37	155	1·24	257·2	19·63	48	175	3·22	295·3	82·66
38	164	1·35	264·0	30·77	49	174	3·62	296·1	87·52
39	167	1·63	271·6	51·16	50	174	3·30	296·8	91·18
40	170	1·98	277·1	54·06	51	174	2·89	297·0	92·30
41	170	2·20	282·1	57·61	52	174	3·06	297·4	93·87
42	171	2·29	284·5	64·36	53	175	3·81	297·6	96·03
43	171	2·36	288·7	67·79	—	Residue	0·61	309·4	92·47
						Total	47·57		

difference is in the very high content of palmitic acid (about 6% more) and low amount of linoleic acid (6·3% less) in cow colostrum fat. Further, both colostral and mature human milk fat contain C_{22} and C_{24} unsaturated acids which are not found in either milk fat or colostrum fat secreted by the cow.

DISCUSSION

The analytical results indicate definite trends in the change in composition of the fat secreted by the cow as the period of lactation progresses. The change in composition of the fat secreted from the first day of lactation onwards is a gradual one. The component fatty acids are different from those of mature milk fat. In this respect the results confirm the findings of Baldwin & Longenecker (1944*a*) that there is difference in the composition of mature and colostrum fat obtained from human milk. But the same authors (1944*b*) later reported that the fatty acid of cow colostrum fat is similar in composition to the typical butter fat as given by Hilditch & Longenecker (1938). Our findings do not agree with the observation made by these workers. The cow colostrum fat analyzed by them was obtained from a single animal, collected moreover during the first 4 days after parturition. We have analyzed composite samples of fat collected from six cows during the progress of lactation, and it may well be that if they had analyzed mature milk fat from the same cow the difference in composition might have been apparent. This change in composition of colostrum fat observed by us cannot be attributed to dietary effects since all the animals received the same diet during the period under observation.

At the start of lactation the cow probably uses her reserves for milk production, but whether these reserves are used specifically to synthesize fat or to furnish energy is unknown. Peterson, Palmer & Eckles (1929) indicate that in the process of milk secretion a fat intermediate in character is first deposited in the glandular tissue and then modified to form milk fat itself. The short chain acids in

Table 3. *The component fatty acids and esters of cow colostrum fat (first day of lactation)*

Acids	% as methyl esters				Fatty acids (excluding unsaponifiable)	
	Lower	Solid	Liquid	Total	% (wt.)	% (molar)
Saturated						
C_4	2·38	—	—	2·38	2·2	6·3
C_6	0·02	—	—	0·02	Trace	Trace
C_8	0·87	—	—	0·87	0·9	1·5
C_{10}	1·24	—	—	1·24	1·2	1·8
C_{12}	1·38	—	0·04	1·42	1·4	1·8
C_{14}	3·52	0·86	5·06	9·44	9·5	10·4
C_{16}	0·75	27·89	1·79	30·43	29·7	29·0
C_{18}	—	10·90	2·57	13·47	13·7	12·1
C_{20}	—	0·61	—	0·61	0·6	0·5
Total	10·16	40·26	9·46	59·88	59·2	63·4
Unsaturated						
C_{10}	0·08	—	—	0·08	0·1	0·1
C_{12}	0·06	—	—	0·06	0·1	0·1
C_{14}	0·29	—	0·54	0·83	0·8	0·9
C_{16}	0·10	—	3·01	3·11	3·0	3·0
C_{18}	—	1·48	31·98	33·46	34·1	30·3
C_{18}	—	—	1·48	1·48	1·5	1·3
C_{20-22}	—	—	1·10	1·10	1·2	0·9
Total	0·53	1·48	38·11	40·12	40·8	36·6
Sum of saturated and unsaturated acids	10·69	41·74	47·57	100·00	100·0	100·0

milk fat are probably derived, as Hilditch first suggested, from the breakdown of some of the long chain acids in the blood fat. It is not clear whether the same process occurs at the initial stages of lactation. It may be that the lower acids are used in the synthesis of oleic acid, but during the first few days of lactation, when the gland is producing much less than the usual amount of fat, the smaller amount of synthesis taking place is able to proceed to its final stages, so that lower acids are almost wholly converted into oleic acid. It is in any case evident that, whatever the mechanism, the mammary gland takes some days to synthesize normal butter fat with the result that the first day's colostrum fat possesses a composition intermediate in character between body fat and milk fat.

SUMMARY

1. Samples of colostrum fat collected from six cows during the progress of lactation have been analyzed for chemical characteristics and the composite mixtures were subjected to fractionation in order to study the fatty acid composition.

2. The results indicate that there is a gradual decrease in the oleic acid, and an increase in the fatty acids of low molecular weight in the colostrum fat as lactation advances.

3. The colostrum fat differs in composition from normal butter fat.

Table 4. *Summary of the component fatty acids of cow colostrum fat (expressed in weight and molar percentages)*

Acids	Weight percentages Days in lactation						Molar percentage Days in lactation					
	1	2	3	4	5	10	1	2	3	4	5	10
Butyric	2·2	2·4	2·6	2·8	3·6	3·6	6·3	6·7	7·1	7·6	9·6	9·9
Caproic	—	0·5	0·8	1·5	0·6	0·6	—	1·0	1·6	3·0	1·2	1·3
Caprylic	0·9	1·2	1·8	2·0	1·8	1·4	1·5	2·5	3·0	3·2	3·0	2·3
Capric	1·2	1·8	2·6	2·3	2·0	1·9	1·8	2·6	3·7	3·2	2·7	2·5
Lauric	1·4	1·8	2·0	2·7	2·6	2·6	1·8	2·2	2·2	3·2	3·1	3·1
Myristic	9·5	9·5	10·3	10·7	11·1	10·8	10·4	10·2	10·9	11·2	11·6	11·3
Palmitic	29·7	32·1	30·5	29·0	30·3	29·4	29·0	30·6	28·6	26·8	28·1	27·3
Stearic	13·7	12·5	12·0	12·6	11·6	12·9	12·1	10·8	10·2	10·5	9·7	10·8
Arachidic	0·6	0·7	0·9	0·8	0·7	1·2	0·5	0·5	0·7	0·6	0·5	0·9
Total	59·2	62·5	63·5	64·4	64·3	64·4	63·4	67·1	68·0	69·3	69·5	69·4
Decenoic	0·1	0·1	0·2	0·2	0·2	0·2	0·1	0·2	0·3	0·3	0·3	0·2
Do-decenoic	0·1	0·1	0·1	0·2	0·3	0·4	0·1	0·2	0·2	0·3	0·3	0·5
Tetra-decenoic	0·8	0·8	0·9	1·1	1·2	0·8	0·9	0·8	0·9	1·1	1·3	0·8
Hexa-decenoic	3·0	3·0	4·7	4·7	3·3	3·5	3·0	2·8	4·4	4·4	3·0	3·3
Oleic	34·1	31·0	28·2	27·5	28·8	29·3	30·3	26·8	24·0	23·1	24·2	24·7
Linoleic	1·5	1·7	1·6	0·8	0·5	0·4	1·3	1·5	1·5	0·7	0·4	0·3
C_{20-22} unsat.	1·2	0·8	0·8	1·1	1·4	1·0	0·9	0·6	0·7	0·8	1·0	0·8
Total	40·8	37·5	36·5	35·6	35·7	35·6	36·6	32·9	32·0	30·7	30·5	30·6
Sum of acids up to C_{14}	16·2	18·2	21·3	23·5	23·4	22·3	22·9	26·4	29·9	33·1	33·1	31·9

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The Capsular Substance of *Bacillus anthracis*

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Iványovics and his colleagues (Iványovics & Erdös, 1937; Brückner & Iványovics, 1937; Iványovics & Brückner, 1937, 1938), following up the earlier work of others (Kramar, 1922; Tomcsik & Bodon, 1934), isolated the capsular substance from capsulated strains of *Bacillus anthracis* and some related organisms in a state approaching purity; the material was shown to have the properties of a hapten, giving precipitates with anti-sera to capsulated strains of *B. anthracis* but failing to induce antibody formation or to confer protection against infection with *B. anthracis* when injected into animals. On hydrolysis, the capsular substance gave good yields of *d*-(−)-glutamic acid, enantiomorphous with the *l*-(+)-acid normally encountered in proteins, and Iványovics & Brückner (1937, 1938) concluded that it was a polypeptide built up solely of *d*-(−)-glutamic acid residues. The work described in the present paper had as its immediate object the elucidation of the detailed chemical structure of this material.

Our work has mostly been carried out with two capsulated strains of *B. anthracis*, viz. 'Vollum', a highly virulent strain originally obtained from Dr R. L. Vollum of the Sir William Dunn Institute of Pathology, Oxford, and 'HM', a non-sporing variant of low virulence obtained by Dr Bruce White, F.R.S., of the National Institute for Medical Research, Hampstead, by growing 'Vollum' in Sclavo serum (see Appendix).

Isolation of crude capsular material

After some preliminary trials the two following methods were found convenient for the isolation of the crude capsular substance:

(*a*) *Cultures grown on agar* are reaped, suspended in water and killed by autoclaving (45 min. at 115°). The autoclaved suspension is strongly acidified (HCl) and treated with 3 vol. of ethanol; after removing bacterial debris by filtration or centrifugation, the crude capsular substance is precipitated from the filtrate by bringing to pH 8–9 with NaOH. (This procedure is a development of a method, fully described in the Appendix, originally devised by Dr Bruce White.)

(*b*) *Cultures grown in liquid media* are autoclaved and centrifuged or filtered. The filtrate is treated with 10% $CuSO_4$ solution and the precipitated copper salt collected by centrifugation; the washed salt is dissolved in acid and decomposed with H_2S. The filtrate from the CuS is adjusted to pH 8–9 and precipitated with ethanol (3 vol.).

Crude materials so prepared from the Vollum and HM strains (and also from another strain, '99', of American origin) were serologically active, giving precipitates with the appropriate anti-serum at dilutions down to 1 in 10^7. These crude preparations give extremely viscous solutions in water, and the best of them probably approximate very closely to the capsular substance as it occurs attached to the organism.

Further purification of capsular substance

Further purification of the capsular substance without extensive degradation is not easy. Dialysis in acid solution is not wholly satisfactory since the molecular weight, calculated from the amino-nitrogen content on the assumption that each molecule contains only one free amino-group, decreases considerably during the process, e.g. the amino-nitrogen content of a crude preparation was doubled by keeping at 25° for 48 hr. in solution at pH 2. The most satisfactory method of purification is by way of the silver salt, which is precipitated

when $AgNO_3$ is added to a solution of the crude capsular substance. The silver salt so obtained is washed, suspended in water and shaken with an excess of the appropriate chloride (NaCl for the preparation of the sodium salt, HCl for the free acid, etc.); AgCl is removed by filtration and the filtrate is dialyzed. In the preparation of the sodium salt dialysis is first carried out, until all chloride ion has been removed, against distilled water with NaOH added to bring the pH to 8–9; the product is then dialyzed against distilled water until its pH falls to 7. In the preparation of the free acid the filtrate is simply dialyzed against distilled water until chloride-free; some degradation under these necessarily acid conditions is unavoidable and the dialysis should not be unnecessarily prolonged. The dialyzed solutions are concentrated to small bulk *in vacuo* at room temperature and finally dried, *in vacuo* over P_2O_5, from the frozen state. The materials so obtained are brittle glasses which dissolve in water, with preliminary swelling, to give viscous solutions; the purified materials are about twice as active serologically as the crude products.

Analytical data

Analytical data on various preparations obtained in this way are given in Tables 1 and 2. These analytical figures are all in agreement with those calculated for a glutamic acid polypeptide containing water not removed under the necessarily mild conditions of drying (P_2O_5 at room temperature); both sulphur and phosphorus are absent from our preparations. The products all give the pink staining reaction with Loeffler's methylene blue shown by the native capsular substance, and even the most highly degraded material (H213B) was serologically active (precipitin reaction with anti-capsular serum down to 1 in 2×10^7). The molecular weights are calculated on the assumption that the molecule contains only one free amino-group; the calculations would be invalidated by pyrrolidone ring formation, but the absence of this disturbing factor is rendered probable by the qualitative observation that the viscosity of the aqueous solution generally runs parallel with the calculated molecular weight. The variations in molecular weight seem to depend on the time of contact with acid. Nearly

Table 1. *Analytical data for the capsular substance (sodium salt) of* B. anthracis

Prep. no.	C %	H %	N %	Na %	NH_2-N* %	C : H : N : Na ratio	Mol. wt. (from N/NH_2-N)	No. of glutamic residues (from N/NH_2-N)	Remarks
H228B	36·4	4·9	8·3	13·3	0·024	5·1 : 8·3 : 1·0 : 1·0	53,000	350	From HM strain
F9	34·6	5·8	8·0	13·0	0·040	5·05 : 10·2 : 1·0 : 1·0	30,000	200	From HM strain
C187A	34·45	4·8	8·1	13·6	0·045	5·0 : 8·3 : 1·0 : 1·0	27,000	180	From HM strain
C187B	32·7	4·8	8·5	12·75	0·061	4·5 : 8·0 : 1·0 : 0·9	21,000	140	From HM strain
F6	33·35	4·9	7·6	15·0	0·076	5·1 : 9·1 : 1·0 : 1·2	15,000	100	From Vollum strain
H228A	37·1	5·4	8·4	11·3	0·085	5·0 : 9·0 : 1·0 : 0·8	15,000	100	From Vollum strain
F4A	37·95	5·9	8·4	11·9	0·092	5·3 : 9·8 : 1·0 : 0·9	14,000	90	From HM strain (purified through Cu salt)
—	39·7	4·0	9·3	15·2	0	5 : 6 : 1 : 1	∞	∞	Theoretical for anhydrous Na salt $(C_5H_6O_3NNa)_\infty$

* Van Slyke; 4 min. reaction time. Longer reaction times give higher values owing to degradation (see p. 300). For this reason all the calculated molecular weights and chain lengths are *minimal* values.

Table 2. *Analytical data for the capsular substance (free acid) of* B. anthracis

Prep. no.	C %	H %	N %	Ash %	NH_2-N* %	C : H : N ratio	Mol. wt. (from N/NH_2-N)	No. of glutamic residues (from N/NH_2-N)	Equiv.	Remarks
H230B	44·0	6·1	10·3	1·2	0·064	5·0 : 8·3 : 1·0	21,000	160	145	From HM strain
F2	44·2	6·1	11·0	0·6	0·116	4·7 : 7·8 : 1·0	12,000	95	140	From HM strain
F5	—	—	9·8	1·05	0·126	—	10,000	80	141	From Vollum strain
F2A	44·95	6·2	9·8	2·6	0·145	5·3 : 8·9 : 1·0	8,700	70	137	From HM strain
H213B	38·2	6·5	9·5	2·5	0·280	4·7 : 9·5 : 1·0	4,500	35	—	From HM strain
—	—	—	9·5	2·9	0·20	—	6,000	48	166	Ivánovics & Brückner (1937). Mean values
—	46·4	5·4	10·9	0	0	5 : 7 : 1	∞	∞	129	Theoretical for $(C_5H_7O_3N)_\infty$

* Van Slyke; 4 min. reaction time. Longer reaction times give higher values owing to degradation (see p. 300). For this reason all the calculated molecular weights and chain lengths are *minimal* values.

all our preparations are less degraded than those of Iványovics & Brückner (1937); the high molecular weight H 228 B is comparable with that of serum albumin (67,000) and higher than those of accepted proteins such as ovalbumin (41,000) and insulin (35,000), and the capsular substance might legitimately be regarded as a protein of particularly simple composition.

The amino-acid composition of the capsular substance

Having thus developed satisfactory procedures for the isolation and purification of the capsular substance, we turned our attention to the problem of its amino-acid composition. Iványovics & Brückner (1937) obtained 86 and 70% yields of *d*-(−)-glutamic acid by the hydrolysis of capsular material obtained from *B. mesentericus* and *B. anthracis* respectively; Bovarnick (1942) reported that 91–97% of the total nitrogen of capsular material from *B. subtilis* was present as glutamic acid, but, since his material was only obtained in solution, these results can only be accepted with reserve.

It is an easy matter to isolate *d*-glutamic acid from the capsular substance (a convenient preparative procedure is described in the experimental section), and there can be no doubt that *d*-glutamic acid is the major constituent of the capsular substance; it is important, however, to demonstrate as completely as possible the absence of any other amino-acids.

Exclusion of amino-acids other than glutamic acid. We therefore attempted to separate dibasic amino-acids quantitatively from monobasic amino-acids by means of amide-exchanging synthetic resins (Cannan, 1944) with a view to applying the method to hydrolysates of the capsular substance; we were able to effect a separation with both Amberlite IR-4 (as used by Cannan) and De-acidite B (from The Permutit Co.), but the results were not wholly satisfactory, the separation being only partial and the resins themselves yielding variable and not inappreciable amounts of nitrogen under the conditions of the separation. We therefore turned to the use of partition chromatography (Martin & Synge, 1941; Gordon, Martin & Synge, 1943). Using the procedure of these workers, almost identical yields of glutamic acid (42·5 and 44·5% respectively) were obtained from hydrolyzed capsular material and authentic glutamic acid subjected to the same experimental procedure; this experiment showed conclusively that the following amino-acids are absent from the capsular substance: phenylalanine, leucine, isoleucine, norleucine, tryptophan, valine, norvaline, methionine, proline, alanine and tyrosine. Cystine, too, must be absent since the capsular substance contains no sulphur.

Estimation of glutamic acid content. From the observed band-rate ($R = 0{\cdot}12$–$0{\cdot}14$) of acetylglutamic acid in 17% butanol-chloroform, it is a simple matter to calculate the partition coefficient between this solvent and water (cf. Martin & Synge, 1941); the value so obtained ($\alpha = 45$) explained our low recovery of glutamic acid. The recovery to be expected with this unfavourable partition coefficient, using the volumes of solvent recommended by Gordon *et al.* (1943), is about 50%; we found 40–45%, and the discrepancy may be due to the formation of some neutral material (possibly 5-acetyl-pyrrolidine) during the acetylation procedure. Clearly the method of Gordon, Martin & Synge is not applicable without modification to glutamic acid and other amino-acids whose acetyl-derivatives have such low band-rates. The difficulty can be overcome by using acetic anhydride in boiling water (cf. Behr & Clarke, 1932) for the acetylation; the acetylation mixture can then be evaporated and the acetyl-amino-acids transferred to the column without the necessity of extracting from aqueous solution. This procedure, which may prove useful for other amino-acids, is not completely satisfactory for glutamic acid owing to the formation of a mixture of acetylglutamic acid and pyrrolidone-carboxylic acid.

We finally decided to convert the glutamic acid into pyrrolidone-carboxylic acid. Wilson & Cannan (1937) have worked out the conditions of temperature and pH necessary for rapid and complete cyclization, and this conversion has been employed by various workers as a basis for methods of estimating glutamic acid; thus Olcott (1944) measured the decrease in amino-nitrogen. Such methods are non-specific, but a specific method results if the pyrrolidone-carboxylic acid formed is estimated by partition chromatography; we have worked out such a method which has given excellent results with the capsular substance and which is now being applied to other proteins. The evaporated protein hydrolysate (a mixture of amino-acid hydrochlorides) is treated with an amount of 0·1 N-NaOH equivalent to the combined HCl present; the solution is brought to pH 2·0 with acetic acid, heated to 130° for 4 hr. in a sealed tube and then evaporated to dryness in a vacuum desiccator over KOH. The pyrrolidone-carboxylic acid in the evaporated product is transferred to a silica-gel column with 17% butanol-chloroform, giving a band ($R = c.\ 0{\cdot}3$) which is eluted and titrated. The residue remaining after transference of the pyrrolidone-carboxylic acid to the column contains the other amino-acids, which can then be estimated by acetylation and partition chromatography. Application of this procedure to authentic *l*-(+)-glutamic acid hydrochloride gave a mean recovery of 95·6% as pyrrolidone-carboxylic acid; in what follows the appropriate correction factor (1·045) has been applied to determinations of glutamic acid by this method. The method is

rapid and convenient; it is easily applicable to 20 mg. of material, and about 0·2 mg. of glutamic acid can readily be detected and estimated in this amount of material if a good sample of silica gel is used.

The capsular substance composed of d-(−)-glutamic acid units. Two preparations of capsular substance (one from the HM strain, the other from the Vollum strain) were hydrolyzed with HCl. The analytical data obtained for the evaporated hydrolysates are

Table 3. *Analytical data on capsular substance hydrolysates*

	F9 (from HM) (%)	H 228 A (from Vollum) strain	
		Exp. I (%)	Exp. II (%)
Total N	8·21	8·46	8·20
Amino-N	8·12	8·22	7·92
Amide-N	0·00	0·15	0·19
Glutamic acid-N	8·06; 8·26	8·22	8·00

given in Table 3. The residual materials, after transfer of the pyrrolidone-carboxylic acid to the column, were acetylated with acetic anhydride in boiling water and subjected to partition chromatography. Only two very small bands ($R = 0·29$ and $R = 0·12$) were detected, and even these were so small that they disappeared before reaching the bottom of the column; their band rates are consistent with pyrrolidone-carboxylic acid and acetylglutamic acid derived from uncyclized glutamic acid. The absence of basic amino-acids and of hydroxyproline was confirmed by the failure of either hydrolysate to give precipitates with phosphotungstic acid or Reinecke's salt. Of all the other amino-acids known to occur in proteins, only glycine ($R = 0·15$ for acetylglycine) and aspartic acid ($R = 0·16$ for acetylaspartic acid) would give bands moving at rates between $R = 0·1$ and $R = 0·3$; the presence of minute traces of one or both of these amino-acids has not been rigorously excluded. However, the glutamic acid estimations account for 99 % of the non-amide nitrogen; the discrepancy is well within the estimated experimental error, and it may confidently be concluded that the capsular substance from both the HM and Vollum strains of *B. anthracis* is built up solely from d-(−)-glutamic acid units.

The structure of the capsular substance

The ready fission of the capsular substance by acid suggested that the glutamic acid residues were joined together, in part at least, by linkages more susceptible to acid hydrolysis than normal α-peptide linkages; further evidence for this view was obtained from Van Slyke amino-nitrogen determinations. It was noticed that the apparent amino-nitrogen content increased with increasing reaction time; in Fig. 1 the apparent molecular weight, calculated from the amino-nitrogen values, is plotted against the reaction time in the Van Slyke apparatus. It will be seen that the effect of varying the reaction time is greatest with specimens of high molecular

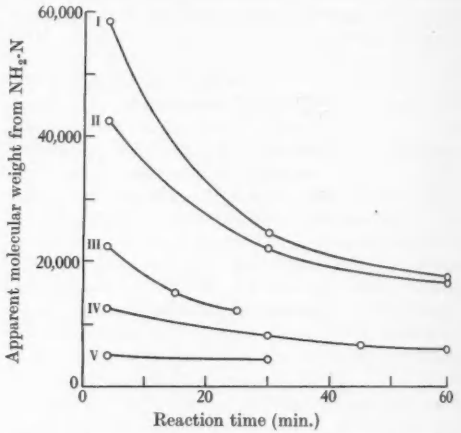

Fig. 1. Effect of varying Van Slyke reaction times on apparent mol.wt. of capsular substance as calculated from amino-nitrogen content. Curve I, H 228 B; curve II, F 9; curve III, H 215 A; curve IV, F 4 A; curve V, H 213 B.

weight and that the apparent molecular weight approaches a limiting value. It is known that glutamine and its *N*-alkyl derivatives yield 2 mol. of nitrogen in the Van Slyke procedure (Chibnall & Westall, 1932; Lichtenstein, 1942), and that glutathione, a γ-peptide of glutamic acid, liberates part of its peptide nitrogen under these conditions; we interpret our Van Slyke results as indicating that, in addition to the free amino-group, the capsular substance also contains γ-peptide groupings which liberate their nitrogen, under the Van Slyke conditions, rather more slowly. The molecular weights given in Tables 1 and 2 are based on amino-nitrogen values obtained with a reaction time of 4 min. and are probably too low owing to some reaction of γ-peptide nitrogen during this period; the error will be greatest in the case of the materials of highest molecular weight. It is noteworthy that one specimen, H 213 B, of apparent mol. wt. 5000, showed only a slight increase in amino-nitrogen with increased reaction time; this specimen probably represents a final degradation product with only α-peptide linkages, all the readily broken γ-linkages having been hydrolyzed by acid during the preparation. It is probable that Iványics' materials of mol. wt. 6000 resemble H 213 B in this respect.

In any polypeptide of glutamic acid made up of $(m+n+1)$ glutamic acid residues linked together

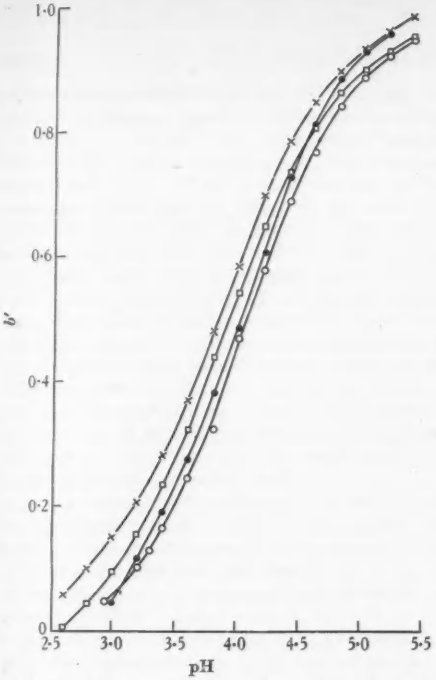

Fig. 2. Titration curves for capsular substance in water. ×——× H228B. ○——○ H230B. □——□ H228A. ●——● F9.

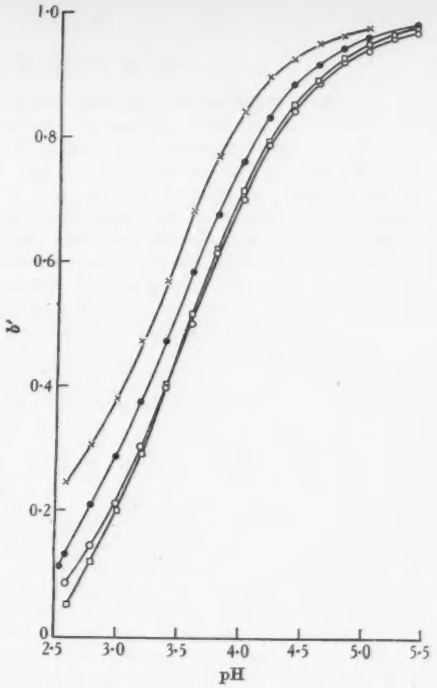

Fig. 3. Titration curves for capsular substance in N-KCl. ×——× H228B. ●——● F9. ○——○ H230B. □——□ H228A.

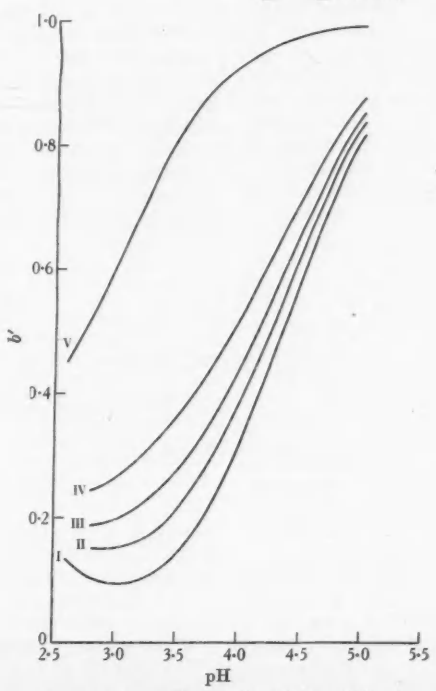

Fig. 4. Theoretical titration curves for capsular substance (H228B) in N-KCl. Curve I, $\alpha/\gamma = 0$; curve II, $\alpha/\gamma = 1/8$; curve III, $\alpha/\gamma = 1/4$; curve IV, $\alpha/\gamma = 1/2$; curve V, $\alpha/\gamma = \infty$.

by m α-peptide linkages and n γ-peptide linkages, there are $(m+n+2)$ free carboxyl groups, of which $(m+1)$ are γ-carboxyls and $(n+1)$ α-carboxyls. The dissociation constants of these two types of carboxyl group are different (in our calculations we have used the values, pK = 4·35 for γ-carboxyl and pK = 2·90 for α-carboxyl, obtained by Mr F. Call by electrometric titration of glutamyl-glutamic acid prepared by the method of Bergmann & Zervas, 1932) and it should be possible to determine the ratio of α- to γ-carboxyl groups by titration experiments. We accordingly carried out electrometric titrations with three preparations of the capsular substance with the results given in Table 4. The

Table 4. *Dissociation constants and ratio of α- to γ-carboxyl groups for the capsular substance of* B. anthracis

Preparation	Mol. wt.	No. of glutamic residues	pG' (in H_2O)	α/γ ratio
H 228 B (Na salt from HM)	53,000	350	3·84	1/8
F 9 (Na salt from HM)	30,000	200	4·02	1/10
H 230 B (Free acid from HM)	21,000	160	4·06	1/15
H 228 A (Na salt from Vollum)	15,000	100	3·93	1/50

titration constants, pG', determined in water, lie very close to the pK value for a γ-carboxyl group, indicating that γ-carboxyl groups preponderate in the capsular substance. Unfortunately it is not possible to evaluate the α/γ ratio by such simple titrations, for two reasons; in the first place, the salt concentration rises throughout the titration, and in the second place the mutual interference of neighbouring carboxyl groups is considerable.

We are indebted to Dr G. S. Hartley for pointing out that these difficulties could be overcome by working in N-KCl solution. Under these conditions the salt concentration clearly remains practically constant throughout the titration. In addition, neighbouring carboxyl groups will not influence one another if the KCl concentration is sufficiently high, since they will be locally neutralized by ionic atmospheres resulting from a redistribution of the K^+ and Cl^- ions; in N-KCl the ionic atmosphere radius is about 3·1 A., whereas the separation of adjacent carboxyl groups in the polypeptide is about 10 A., and the conditions for non-interference are thus fulfilled sufficiently well in N-KCl solution. In N-KCl solution the titration curves were, of course, considerably displaced; the α/γ ratios were evaluated by plotting the titration curves (pH against $b' = \alpha + h/c$; Simms, 1926; von Muralt, 1930) on tracing paper and fitting them as closely as possible to theoretical curves calculated for various α/γ ratios by means of the accurate modified Henderson equation, $pH = pK + \log b'/(1-b')$. Although a very high degree of accuracy cannot be claimed for the α/γ ratios so evaluated, there is clearly a correlation between the molecular weight and the α/γ ratio; preparations of low molecular weight contain a higher proportion of γ-carboxyl groups (and hence a lower proportion of γ-peptide linkages) than preparations of high molecular weight. This is the same conclusion as was reached by considering the amino-nitrogen results.

On the basis of this evidence we conclude that the capsular substance is built up from α- and γ-polypeptide chains of *d*-(−)-glutamic acid units. The high viscosity of solutions of the capsular substance, as compared with solutions of globular proteins of similar molecular weights, clearly indicates that the molecules are thread-like in shape; it seems probable, therefore, that the individual α- and γ-peptide chains are linked together at, or near, their ends. Accordingly we suggest a repeating structure (I) for the capsular substance (Fig. 5).

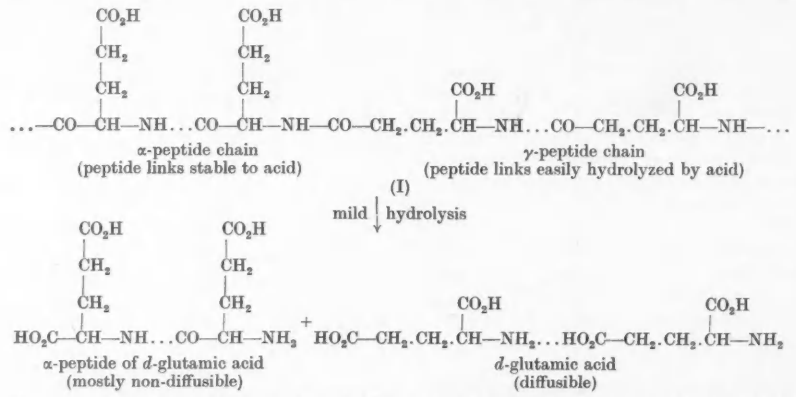

Fig. 5. The repeating structure of the capsular substance of *B. anthracis* and formulation of its hydrolytic fission.

It will be seen that the behaviour to be expected of such a substance on mild hydrolysis (involving the fission of γ-, but not α-, peptide linkages), followed by dialysis, is precisely that which was observed during the purification of the polypeptide, viz. conversion into a product of lower average molecular weight with a smaller proportion of free α-carboxyl groups.

A further quantitative experiment was carried out in which a specimen (H 230 B) of the capsular substance was dialyzed for 7 days at room temperature in 0·01 N-HCl. The experimental findings agreed with those expected for a substance of structure I (Fig. 5); about 3% of the nitrogen (representing that contained in γ-peptide linked glutamic acid residues) was found to have diffused through the membrane. 60% of this nitrogen was amino-nitrogen, indicating that the diffusible material was predominantly glutamic acid with a little peptide. The N/NH_2-N ratio of the indiffusible portion indicated a chain length of 90 glutamic acid units as compared with 160 units for the original specimen; clearly the individual α-peptide chains are themselves quite long.

It remains only to add that we have encountered no valid evidence for any chemical difference between the capsular substances produced by the two strains of *B. anthracis* used in this work.

EXPERIMENTAL

The bacterial suspensions used in this work were grown for us by Dr G. P. Gladstone by the following methods:

(i) '*Vollum*' *strain.* This strain is not normally capsulated when grown on the ordinary laboratory media, but a heavy growth of capsulated organisms can be obtained in broth cultures by adequate aeration with air containing 5–20% CO_2. For the present work the organism was grown on the acid-hydrolyzed casein—yeast extract broth ('CCY') of Gladstone & Fildes (1940).

1 l. of CCY broth was inoculated with 1 ml. of a spore suspension containing about 10^8 spores; after growing stagnant for 6 hr. this culture was added to a further litre of CCY broth in an apparatus fitted for vigorous aeration through a sintered glass disk (porosity 4). Growth was then continued at 37° for a further 16 hr. while air containing 10% CO_2 was passed through at 500 ml./min. The culture so obtained was microscopically homogeneous, consisting entirely of short chains of large, heavily capsulated bacilli; no spores were seen.

(ii) '*HM*' *strain.* This strain gives a viscous mucoid growth, showing well-defined capsules, on ordinary media (see Appendix); there is a tendency for capsule production to be lost on subculture, and repeated plating and selection of mucoid colonies was necessary. The method of growth was similar to that used by Henderson & Morgan (1938) for *Eberthella typhosa*.

Enamel photographic dishes, 10 × 14 × 1 in., each containing 500 ml. of CCY agar, were inoculated with a suspension of an 18 hr. culture by means of a glass spreader. The thick viscous growth, consisting of about 90% capsulated bacilli in chains, was scraped off with a glass plate after 18 hr. at 37° and suspended in water (25 ml. per dish).

The serological tests were also carried out by Dr G. P. Gladstone, who has supplied the following information. Two anti-sera were used, one obtained by immunizing rabbits with capsulated organisms of the Vollum strain killed with 0·001 M-$HgCl_2$, the other by immunizing rabbits with Vollum spores killed by autoclaving (it has been found that small amounts of capsular substance can be extracted from washed Vollum spores by the procedure described below). The preparation of such anti-capsular sera is difficult and erratic; full details will be published elsewhere.

The precipitation tests were carried out by mixing these sera, diluted 1 in 8, with equal volumes of serial dilutions of the capsular substance in saline. Tests were read after incubation at 37° for 1 hr. followed by keeping overnight in the refrigerator. As there was no evidence of solubility of the precipitate in excess of antiserum, readings at the end-point were considered to give an approximate indication of the amount of capsular substance present.

Isolation and purification of the capsular substance

Preliminary experiments. The experiments outlined in Table 5 were carried out with the HM strain; the organism was killed by heating to 60°. After the preliminary treatment outlined in the Table, the suspension was treated with HCl until it no longer clotted on the addition of 3 vol. of ethanol; 3 vol. of ethanol were then added and the suspension was filtered through kieselguhr or separated on the Laval centrifuge. The clear filtrate was brought to pH 8–9 with NaOH; after settling for some hours the precipitate was collected by decantation and centrifugation, washed with absolute ethanol and dried in a vacuum desiccator.

The products were all serologically active, giving precipitin reactions with anti-capsular serum down to $1 : 10^7$. All the preparations were carbohydrate free (Molisch reaction); spectroscopic examination showed the presence of large amounts of Na, Ca and Mg together with small amounts of other metals.

Clearly, simple autoclaving (process xi) was the simplest and most efficient and was adopted for the routine preparation of the capsular substance.

Recommended procedure. The following details apply to the sodium salt; other salts, or the free

Table 5. *Preliminary experiments on the isolation of the capsular substance of* B. anthracis

Procedure	Vol. of suspension used (ml.)	Yield mg.	Yield mg./ml.	Prep. no.
(i) Suspension diluted with equal vol. water. Adjusted to pH 8·5. Refluxed for 3 hr.	100	600	6·0	H208A
	1,000	6,860	6·9	R519A
	2,000	13,720	6·9	H217B
(ii) Suspension diluted with 3 vol. water. Adjusted to pH 8·5. Heated 4 hr. on boiling water-bath	200	910	4·6	H207E
(iii) Suspension diluted with equal vol. water. Adjusted to pH 9·5. Incubated at 37° for 20 hr. with 500 mg. trypsin	100	470	4·7	H216C
(iv) Suspension adjusted to pH 3–4 with HCl. Treated with 3 vol. EtOH. Shaken 18 hr.	500	3,600	7·2	R519B
(v) Suspension adjusted to pH 3–4 with HCl. 2·5 g. Na tauroglycocholate in 20 ml. water added. Kept 1 hr. at room temp.	500	2,310	4·6	R519C
(vi) Suspension brought to pH 3–4 with HCl. Kept 1 hr. at room temp.	500	3,190	6·4	H211C
(vii) Suspension diluted with equal vol. water and brought to pH 8·5. 0·1% Aerosol OT added. Kept 24 hr. at room temp.	60	220	3·7	R516A
	60	180	3·0	H209A
(viii) Suspension diluted with 3 vol. water. Brought to pH 8·5. 1% Aerosol OT added. Kept 24 hr. at 5°	50	200	4·0	R515A
(ix) Suspension brought to pH 3–4 with HCl. 0·5% Aerosol OT added. Kept 1 hr. at room temp.	500	3,710	7·4	H211B
	3,000	13,900	4·6	H215A
	1,000	5,840	5·8	H217A
(x) Suspension diluted with equal vol. water and autoclaved. Adjusted to pH 3–4. 0·5% Aerosol OT added. Kept 1 hr. at room temp.	3,000	15,000	5·0	F3
(xi) Suspension autoclaved only	2,600	21,000	8·1	F9
	3,000	32,000	10·7	F4

acid, are obtained by replacing NaCl by the appropriate chloride in the decomposition of the silver salt.

A thick, autoclaved (120° for 45 min.) suspension of the capsulated organisms is treated with sufficient 5N-HCl to prevent separation of a bulky flocculent precipitate on dilution with alcohol. Ethanol (3 vol.) is then added and bacterial bodies, etc. are removed by centrifugation (Laval) or filtration. Sufficient ethanol is added to the clear filtrate to make up for losses by evaporation during the clarification process and 10% NaOH is added to bring the pH to 9. The precipitated crude polypeptide is collected by decantation and centrifugation. After drying in a vacuum desiccator the crude product is dissolved, with gentle warming, in sufficient water to give a not too viscous solution. After adjusting to pH 7·4 an excess of silver nitrate solution is added (the yellow precipitate flocculates and settles readily when a sufficient excess has been added). The silver salt is filtered off, washed well with water and suspended in an excess of NaCl solution; the mixture is shaken for 30 min. and kept in the refrigerator overnight. AgCl is removed by filtration (finally through kieselguhr) and the filtrate is brought to pH 9 and dialyzed against frequent changes of distilled water, brought to pH 9 with NaOH, until free from chloride. Dialysis is continued against distilled water until the contents of the dialysis bag reach pH 7·5; the dialyzed solution is then evaporated, first under reduced pressure at room temperature and finally from the frozen state over P_2O_5.

The sodium salt of the capsular substances is so obtained as a colourless or pale yellow crisp glass permeated with air bubbles and of reasonably uniform composition; if the final freeze-drying is omitted, the product is a horny mass the moisture content of which is very dependent on the thickness of the film.

Composition of the capsular substance

Preparation of d-*glutamic acid hydrochloride.* Organisms of the HM strain, from growth on 60 agar trays, are suspended in 10 l. of water, autoclaved and separated on the Laval centrifuge. The fluid is adjusted to pH 8 and treated with an excess of 10% copper sulphate solution (*c.* 500 ml.). After allowing to settle overnight the copper salt is filtered off, washed thoroughly with water and refluxed with 6N-HCl (600 ml.) for 2½ hr. After the addition of 200 g. of ice, H_2S is passed in for 10 min. The precipitated CuS is filtered off and the filtrate is concentrated under reduced pressure to about 100 ml. After keeping overnight in the refrigerator, the crude *d*-glutamic acid hydrochloride (20 g.) is collected by filtration, washed with a little cold conc. HCl and dried in a vacuum desiccator; a small second crop can be obtained from the mother liquor by concentration and saturation with HCl. Recrystallization, by saturation of the aqueous solution with HCl, gives, without much

loss, pure *d*-(−)-glutamic acid hydrochloride, m.p. 206°, $[\alpha]_D^{11°}$ −24·2° (*c* = 0·83 in N-HCl) (lit.: m.p. 201°; $[\alpha]_D$ −24·7°).

Chromatographic determination of glutamic acid as pyrrolidone-carboxylic acid. The following experimental procedure has been found satisfactory:

25 mg. of a mixture of amino-acid hydrochlorides (e.g. an evaporated protein hydrolysate) is treated with an amount of 0·1 N-NaOH exactly equivalent to the combined HCl present; 4 ml. of water and 2·5 ml. of acetic acid are added to the solution, bringing it to pH 2. The solution is heated in a sealed tube at 130° for 4 hr. and then evaporated to dryness at room temperature in a vacuum desiccator over KOH. The product is transferred by means of 17% butanol-chloroform to a Synge column made up from 3 g. of suitable silica gel, 1·5 ml. of 0·025% indicator *R*-NH_4 (Liddell & Rydon, 1944) and 3% butanol-chloroform. The blue band of pyrrolidone-carboxylic acid (*R* = *c.* 0·3) is eluted from the column with 17% butanol-chloroform; the solvent is evaporated from the eluate *in vacuo* and the residue titrated with 0·01 N-baryta, using bromthymol blue as indicator.

The details of a typical experiment with *l*-(+)-glutamic acid hydrochloride were as follows:

Material used: 25·0 mg. (0·1362 mmol.).

Pyrrolidone-carboxylic acid: Titre = 13·0 ml. of 0·010 N-baryta.

Therefore recovery = 0·1300 mmol. = 95·4%.

Residue after transference to column: Titre = 0·50 ml. of 0·010 N-baryta.

Therefore loss = 0·0050 mmol. = 3·7%.

In a duplicate experiment the recovery of pyrrolidone-carboxylic acid was 95·8% and the loss in the residue 3·0%.

Hydrolysis of capsular substance from the HM strain. 212·0 mg. of the sodium salt F 9 were heated in a sealed tube at 100° for 18 hr. with 6 N-HCl (24 ml.) and acetic acid (6·4 ml.). The hydrolysate was evaporated to dryness *in vacuo* and kept for several days in a vacuum desiccator over KOH and silica gel. A stock solution was prepared by dissolving the residue in water (total vol. = 10 ml.).

Analysis of this stock solution gave the following results:

Total nitrogen	1·74 mg./ml.
Amino-nitrogen	1·72 mg./ml.
Amide-nitrogen	Nil
Chloride ion	0·250 m-equiv./ml.

1 ml. gave no precipitate with 1 ml. of a saturated solution of Reinecke's salt or with 1 ml. of a 15% solution of phosphotungstic acid in 5% H_2SO_4.

1 ml. was treated with 1·25 ml. of 0·1 N-NaOH (equivalent to the combined HCl, the remainder of the chloride ion being present as NaCl), 3 ml. of water and 2·5 ml. of glacial acetic acid. After heating at 130° for 4 hr., the solution was evaporated and the pyrrolidone-carboxylic acid transferred to a silica-gel column; the single blue band had *R* = 0·30. The evaporated eluate required 11·70 ml. of 0·01 N-baryta for neutralization. This corresponds to 1·64 mg. of nitrogen (uncorrected) or 1·71 mg. (= 98% of the total nitrogen) after correcting for the losses observed with authentic glutamic acid. In a repeat experiment the titre was 12·00 ml. of 0·01 N-baryta, i.e. 1·68 mg. N (uncorrected) or 1·75 mg. N (corrected), corresponding to 101% of the total nitrogen. The residue left after transferring the pyrrolidone-carboxylic acid to the column was dried, dissolved in 3 ml. of boiling water and acetylated by the addition of 1 ml. of acetic anhydride to the boiling solution in small quantities with shaking. The evaporated product was transferred to a silica-gel column with 17% butanol-chloroform; two small bands were observed (*R* = 0·29, corresponding to pyrrolidone-carboxylic acid, and *R* = 0·12, corresponding to acetylglutamic acid) but disappeared before leaving the column; no bands corresponding to other amino-acids could be detected. Control experiments showed that small amounts of acetyl-aspartic acid or acetylglycine would probably have been detected at this stage.

Hydrolysis of capsular substance from the Vollum strain. 208·0 mg. of the sodium salt H 228 A were heated in a sealed tube at 100° for 18 hr. with 6 N-HCl (24 ml.) and acetic acid (6·4 ml.). The hydrolysate was treated as described above and a stock solution (10 ml.) made up. This gave no precipitate with a saturated solution of Reinecke's salt or with 15% phosphotungstic acid in 5% sulphuric acid.

Analysis of the stock solution gave the following results:

Total nitrogen	1·76 mg./ml.
Amino-nitrogen	1·71 mg./ml.
Amide-nitrogen	0·032 mg./ml.
Chloride ion	0·244 m-equiv./ml.

1 ml. of stock solution was subjected to the process for conversion into pyrrolidone-carboxylic acid. The pyrrolidone-carboxylic acid band had *R* = 0·30; titration (required 11·65 ml. of 0·010 N-baryta) showed that the band corresponded to 1·63 mg. (uncorrected) or 1·71 (corrected) of nitrogen; the latter figure accounts for 99% of the non-amide-nitrogen. Acetylation and partition chromatography of the residue gave only one very small band (*R* = 0·12, corresponding to acetylglutamic acid) which disappeared before it left the column.

The following results were obtained in a repeat experiment with another sample of H 228 A; the hydrolysate was made up to 10 ml.:

Total nitrogen	1·025 mg./ml.
Amino-nitrogen	0·99 mg./ml.
Amide-nitrogen	0·024 mg./ml.
Glutamic acid-nitrogen	1·00 mg./ml. = 100% of non-amide-nitrogen

Structure of the capsular substance

Hydrolysis in acid solution. A solution of crude capsular material (C154B; *c.* 10 mg./ml.) was brought to pH 2 with HCl and kept at 25°, samples being removed from time to time for determination of amino-nitrogen. The following results, expressed as % NH_2-N on the original dry weight, were obtained:

Time (days):	0	1	2	7	14
% NH_2-N:	0·055	0·087	0·117	0·167	0·398

Electrometric titrations. The instrument used was a Cambridge pH meter with glass and calomel electrodes.

(i) *Titrations in water.* The substance was dissolved in a known volume of water, containing a known excess of standard acid, and back titrated with 0·1045N-baryta; readings on the pH meter were taken at intervals of 0·10 ml. The solutions were stirred by a current of nitrogen. The observations were plotted and b' (Simms, 1926) was calculated at pH intervals of 0·20; the titration constants ($pG' = $pH when $b' = 0{\cdot}50$) were read off from the curves relating pH to b' which are shown in Fig. 2. The details of a typical experiment are given in Table 6.

(ii) *Titrations in* N-KCl. The technique was similar to that used for the experiments in water, but the solutions were initially made up to be N in KCl, and an equal volume of 2N-KCl was added after each addition of baryta and before taking the pH reading. The details of a typical experiment are given in Table 7 and the pH-b' curves in Fig. 3.

Theoretical titration curves for the case of H228B are shown in Fig. 4. Similar sets of curves were constructed for H230B and H228A but are not reproduced.

Dialysis in acid solution. H230B (116·2 mg.) was dissolved in water (10 ml.) and dialyzed for 7 days at room temperature against 220 ml. of 0·01N-HCl, precautions being taken to prevent ammonia, etc. entering the solutions from the atmosphere. The solutions were then evaporated to dryness, first *in vacuo* at room temperature and finally from the frozen state over P_2O_5. Analysis gave the following results:

	Total N (mg.)	NH_2-N (mg.)	(Total N/NH_2-N) = mean chain length
Dialysate	0·364	0·206	1·8
Residue	11·4	0·126	90

No pyrrolidone-carboxylic acid could be detected in the dialysate by the adsorption-chromatographic method.

Table 6. *Electrometric titration of capsular substance in water*

H228B. 88·7 mg. in 6·00 ml. 0·1003N-HCl and 14·00 ml. water. Na content of H228B = 13·34%. Hence HCl required = 0·5145 m-equiv. Therefore excess HCl added = 0·0873 m-equiv. Equiv. (from titration to pH 7) = 172·3. Temp. = 20°.

pH	Baryta added (ml.)	b	a	h	c	b'
2·35	0·00	0·000000	0·004365	0·00447	0·02573	0·004
2·40	0·16	0·000830	0·004332	0·00398	0·02553	0·031
2·60	0·62	0·003142	0·004234	0·00251	0·02495	0·057
2·80	1·00	0·004976	0·004158	0·00158	0·02450	0·098
3·00	1·38	0·006745	0·004083	0·00100	0·02406	0·153
3·20	1·72	0·008273	0·004020	0·00063	0·02369	0·206
3·40	2·14	0·01010	0·003943	0·00040	0·02324	0·282
3·60	2·60	0·01202	0·003863	0·00025	0·02277	0·369
3·80	3·16	0·01426	0·003770	0·00016	0·02222	0·479
4·00	3·70	0·01631	0·003684	0·00010	0·02171	0·586
4·20	4·20	0·01813	0·003608	0·00006	0·02126	0·699
4·40	4·70	0·01988	0·003534	0·00004	0·02083	0·787
4·60	5·02	0·02097	0·003489	0·000025	0·02057	0·851
4·80	5·26	0·02176	0·003456	0·00002	0·02037	0·900
5·00	5·44	0·02235	0·003432	0·00001	0·02022	0·936
5·20	5·58	0·02279	0·003413	0·00001	0·02011	0·964
5·40	5·70	0·02318	0·003396	0·00000	0·02002	0·989

The symbols at the heads of columns 3–7 have the following significance:

b = concentration (equiv./l.) of added base (baryta),
a = " " of added acid (HCl),
h = " " of hydrogen ion,
c = " " of polypeptide,

$$b' = \frac{b - a + h}{c}.$$

Table 7. *Electrometric titration of capsular substance in* N-KCl

H228B. 85·5 mg. in 6·00 ml. 0·1003N-HCl, 4·00 ml. water and 10·00 ml. 2N-KCl. Na content of H228B = 13·34%. Hence HCl required = 0·4961 m-equiv. Therefore excess HCl added = 0·1057 m-equiv. Equiv. = 172·3. Temp. = 17·5°.

pH	Baryta added (ml.)	*b*	*a*	*h*	*c*	*b'*
1·95	0·00	0·000000	0·005285	0·01122	0·02481	0·239
2·00	0·16	0·000823	0·005201	0·01000	0·02442	0·230
2·20	0·72	0·003510	0·004929	0·00631	0·02314	0·211
2·40	1·22	0·005682	0·004710	0·00398	0·02211	0·224
2·60	1·62	0·007285	0·004547	0·00251	0·02135	0·246
2·80	2·10	0·009067	0·004367	0·001585	0·02050	0·307
3·00	2·58	0·01072	0·004201	0·00100	0·01972	0·381
3·20	3·10	0·01237	0·004033	0·00063	0·01893	0·474
3·40	3·62	0·01389	0·003881	0·00040	0·01822	0·571
3·60	4·18	0·01540	0·003727	0·00025	0·01750	0·682
3·80	4·62	0·01651	0·003614	0·00016	0·01696	0·769
4·00	4·98	0·01737	0·003527	0·00010	0·01656	0·842
4·20	5·26	0·01801	0·003462	0·00006	0·01626	0·899
4·40	5·40	0·01832	0·003431	0·00004	0·01611	0·927
4·60	5·54	0·01862	0·003400	0·000025	0·01596	0·955
4·80	5·60	0·01875	0·003387	0·00002	0·01590	0·967
5·00	5·67	0·01888	0·003375	0·00001	0·01584	0·979

Symbols have same significance as in Table 6.

SUMMARY

1. Methods are described for the isolation and purification, without undue degradation, of the capsular substance from two strains of *B. anthracis*.

2. There is no evidence for any chemical differences between the capsular substances obtained from these two strains.

3. The capsular substance is shown to be made up solely from *d*-(−)-glutamic acid residues; the molecular weight of the native material is greater than 50,000 and it is thus of the same order of size as many proteins.

4. Structurally, the capsular substance is a long-chain molecule made up of α-peptide chains of 50–100 *d*-glutamic acid residues joined together by γ-peptide chains of *d*-glutamic acid residues (see p. 302).

We are greatly indebted to Dr G. P. Gladstone for generous supplies of bacterial cultures and for carrying out the serological tests, to Messrs F. Call, J. E. Cave-Browne-Cave and E. S. J. Fry for much help with some parts of the experimental work, and to Sir Paul Fildes, F.R.S., the Director of the Unit, for his encouragement and interest.

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Appendix. Note on the Selection of Non-sporing and Mucoid Variants from a Strain of *B. anthracis* and on the Extraction from the Mucoid Form of the Capsular Substance

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In the course of some work on *B. anthracis* the problem arose of separating from a classic culture of that organism non-sporing races and races similar to the strains referred to by Tomcsik & Szongott (1933) and Ivánovics & Erdös (1937), showing free development of capsules under ordinary conditions of cultivation *in vitro*. Tentatively I applied to the strain 'Vollum' the method of treatment with activated specific serum which I had employed with success for the isolation of rough and other variants from vibrio cultures. For this enterprise there was available in the first place a sample of a veterinary anti-anthrax serum from Budapest, and later a sample of Sclavo's serum prepared by the Tuscan Serum and Vaccine Institute, Sienna. With slight variations the mixtures prepared were as follows:

	Tubes 1 (*a*, *b* and *c*) (ml.)	Tubes 2 (*a*, *b* and *c*) (ml.)	Tubes 3 (*a*, *b* and *c*) (ml.)	Tubes 4 (*a*, *b* and *c*) (ml.)
Anti-anthrax serum diluted 1 : 50	1	0·5	0·2	0·1
Saline	—	0·5	0·8	0·9
Fresh guinea-pig serum (complement) diluted (with nutrient broth) in series: (*a*) 1 : 5, (*b*) 1 : 10, (*c*) 1 : 15	0·5	0·5	0·5	0·5

To the 1·5 ml. contents of each of the twelve experimental tubes and of controls lacking either complement or specific serum was added a drop of a faintly turbid suspension of a 12 hr. culture of the 'Vollum' strain of *B. anthracis*.

After 12 and 18 hours' incubation at 37° the mixtures were plated out on nutrient agar. In these platings, together with colonies of the classic type there appeared in many instances a number of the circular, raised, opaque, moistly caseous and yellowish colonies of the type which—though 'roughness' is not yet known to occur in this group of bacilli—has sometimes been called 'smooth'. These latter organisms proved to be asporogenous and, though those isolated by the agency of the Budapest serum eventually resumed sporulation in subculture, many of those obtained by use of the Sclavo serum were fixed in their peculiarity and have long been propagated on solid and in liquid media without forming elements surviving 20 min. heating at 60°.

In platings of cultures treated with the Budapest serum there appeared also colonies of mucoid aspect and consistence formed of magnificently capsulated bacilli. The condition was transmissible in subculture but the cultures were apt to contain spores. To obtain races both capsulate and absolute in asporogenicity the attempt was made to select by means of the Budapest serum mucoid races from the non-sporing Sclavo-won Hampstead (or H) variants, which, like the 'Vollum' parent showed in ordinary culture occasional capsulate units. The trial was entirely successful and there were obtained the Hampstead Mucoid (or HM) series of variant races (Plate 4) which have since been maintained in full character by intermittent selection of colonies of mucoid habit.

It may be noted that at first isolation the H variants tested showed virulence for guinea-pigs and mice not markedly less than that of the parent culture while the virulence of the HM variants was from the outset of a much lower order.

Being in total ignorance of the serological factors involved in the somewhat differing action of the two antisera I am unable to point the rationale of the events outlined. It is, however, to be suspected that the variants presented themselves rather through the selective than the stimulant action of the treatment to which the parent culture was subjected. Over the range of the experiments made no special relation between the serum dose employed and the appearance of the variants was observed.

Extraction of capsular material

Extraction of the capsular material, offered in abundance by the HM cultures, was undertaken; it was quickly found that this could be achieved by ethanol containing mineral acid—the solvent previously employed for the extraction of ethanol-soluble proteins from Gram-negative bacilli (White, 1932, 1934, 1936).

The organism was grown in mass in enamelled trays, 24 trays affording 20 sq.ft. of nutrient agar surface. The slimy growth, collected after 24–48 hr. incubation at 37° and killed and dispersed in water by steaming, was diluted till it could be refluxed on a sand bath, which process, possibly unnecessary and even undesirable, was maintained for some hours. A pilot experiment having been made on a small sample of the material, the bulk was mixed with 3 vol. of ethanol and the requisite amount of hydrochloric

Plate 4. Micro-photograph of a young culture of an HM variant of *B. anthracis* ('Vollum') positively stained with methylene blue and negatively stained with congo red. The capsules are shown in relief. In some rods capsulation has not yet developed or is in process of development. Photograph by Mr R. McV. Weston, F.R.M.S., F.R.P.S. (× 1500).

acid was slowly added with stirring to disperse, by dissolution of the capsular substance, the stringy coagulum first formed, leaving a granular precipitate. The ethanolic supernatant, thoroughly filtered, was treated with NaOH solution until with neutralization maximum precipitation had occurred. After re-solution in acid 75% (v/v) ethanol, re-filtration and re-precipitation with NaOH, this precipitate, dissolved in a small volume of water, was subjected to dialysis against N/50 HCl and finally against distilled water—a precipitate forming during dialysis being removed by centrifugation. On evaporation the dialyzed solution became viscid as dryness was approached and the capsular material was eventually left as a tough vitreous layer on the floor of the containing dish from which it was parted with difficulty, emitting at separation an almost metallic 'cry'.

This material showed the general features of the products of Tomcsik and Ivánovics and their collaborators and, though reacting in high dilution with the only anti-P (anti-capsular substance) serum prepared against the whole organism, failed itself to engender a formation of antibodies in any of several rabbits injected with its solutions.

There was observed its ability to form precipitates with methylene blue and atebrin and Dr Harold King noted a similar reaction with diamidines. My colleague, Mrs R. V. Pitt Rivers, who further purified and freeze-dried a sample of the substance, from free amino-N determinations assessed the molecular weight at about ~~9000~~. 22,400

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The Manometric Determination of the Activity of Carbonic Anhydrase under Varied Conditions

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The activity of carbonic anhydrase was first measured by Meldrum & Roughton (1932) with the boat-manometric method which Brinkman, Margaria & Roughton (1933) had developed for study of the kinetics of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ in absence of enzyme. In this method a buffer solution is shaken violently with bicarbonate solution (or with a CO_2-containing gas phase) in a boat-shaped vessel which is flexibly connected to a U-tube manometer. The rate of CO_2 output (or uptake) is followed by reading the pressure at various times after the shaking begins. The magnification of the rate, when carbonic anhydrase is added to the buffer, is taken as a measure of the activity of the enzyme. Variations and improvements in the manometric method have since been made by Stadie & O'Brien (1933), Van Goor (1934), Roughton & Booth (1938), Kiese & Hastings (1940), Kiese (1941), and Leiner (1941).

In all such work diffusion between the gas and liquid phases tends to become a limiting factor as the reaction velocity of the chemical process rises. The influence of diffusion can, however, be allowed for quantitatively by the theoretical and experimental methods recently worked out by Roughton (1941) on the basis of the stationary film theory of gas-liquid interchange.

In another set of methods the time is measured for the reaction to bring about a certain change in pH in absence and in presence of the enzyme. Stadie & O'Brien (1933) followed the pH electrometrically, but most authors have preferred the much more simple methods with indicators (Brinkman, 1933; Van Goor, 1934; Philpot & Philpot, 1936). These methods are, however, in general much less powerful and definite than manometry as regards the range of conditions under which the activity of the enzyme can be investigated.

All the methods hitherto used are fairly adequate for determining the number of enzyme units present in a given preparation and have accordingly been much used in

(i) The study of the distribution of the enzyme in the blood, body fluids and tissues of a wide range of organisms with a view to unravelling its role in CO_2 transport, gastric secretion and other physiological processes.

(ii) The development of methods of purifying the enzyme, culminating recently in its crystallization by Scott & Fisher (1942).

(iii) The study of inhibitors and activators, though here much caution is needed in interpreting the results.

So far, however, no detailed study has been published of

(iv) The physico-chemical kinetics of the enzyme, e.g. the effect of substrate concentration, enzyme concentration, pH, salts, temperature, etc. Such knowledge is indispensable for working out the quantitative role of the enzyme in physiological processes (such as CO_2 removal into the expired air), as well as from the general viewpoint of enzyme chemistry.

Arbitrary units of enzyme activity are of no service for the projects listed under (iv), and it is instead essential to determine, in the ordinary chemical units of mol./l./sec., the extra rate of H_2CO_3 dehydration or CO_2 hydration brought about by the enzyme. This task has, for the following reasons, proved far more cumbrous than is usually the case in enzyme kinetics.

(*a*) CO_2 hydration and H_2CO_3 dehydration proceed at appreciable rates in absence of the enzyme, and furthermore are catalyzed by inorganic buffers (Roughton & Booth, 1938; Kiese & Hastings, 1940).

(*b*) Progressive, and often appreciable, inactivation of the enzyme occurs during the manometric shaking.

(*c*) As mentioned already, the results have to be corrected for the limiting effect of diffusion on the speed of the manometric changes.

We have, however, extended the manometric method to cope with these difficulties, though at the cost of much complexity in the computations. In the following paper we describe the applications of the new procedure to the kinetics of the enzyme. But the extensions which we have made to the existing methods may well be necessary at times in work on the distribution, function, purification, inhibition, destruction and activation of the enzyme, and it therefore seems useful to present them separately here.

EXPERIMENTAL METHODS

For the attainment of accurate and repeatable results under varied conditions we have made some changes in the boat-manometric technique since our previous paper (Roughton & Booth, 1938), which should, however, be consulted for fuller details of the actual procedure. We have also had to take special care with the preparation of the buffer solutions and the handling of the enzyme preparations. Points under these three headings are discussed in the present section.

The boat-manometric technique. In all our recent experiments 4·2 ml. of liquid at 0° have been shaken violently at 290–320 times/min. with a 60 ml. gas phase at the same total air pressure within the manometric system as before, namely $\frac{1}{8}$ atm. for CO_2 output and $\frac{1}{7}$ atm. for CO_2 uptake experiments. In our 1938 technique the 64 ml. experimental vessel was connected to one limb of the manometer, to the other limb of which was connected a 200 ml. thermo-compensating bottle fixed in one corner of the thermostat tank. Even with vigorous stirring of the tank a small variable irregularity traceable to thermal effects was observed in slow experiments. We have, therefore, replaced the fixed compensating bottle by a vessel identical in size and shape with the 'live' experimental vessel and held on the same shaker bar. The whole arrangement of manometric tubes and vessels is now symmetrical (as in the Barcroft differential manometer), and minute thermal and mechanical disturbances tend to balance out. The 'live' vessel is loaded with buffer solution ±enzyme, and with bicarbonate or with CO_2 gas according to whether CO_2 output or uptake is to be measured: the compensating vessel is freshly loaded with the same total volume of liquid (water) as the live vessel for each experiment. The buffer solutions are made up with water previously equilibrated with air at room temperature, and similar water is used in the compensating vessel. An almost equal amount of air therefore comes out of solution in each of the two vessels when shaking begins, so that manometric effects due to gases other than CO_2 are, in this way, practically eliminated.

In our recent work we have replaced the metal thermostat tank by a glass aquarium jar, so as to be able to see more easily inside the tank during necessary manipulations of the vessels and their connexions *in situ*. The ridged two-compartment vessels, which are indispensable for CO_2 output experiments, have been used also for CO_2 uptake so as to make conditions more comparable.

In uptake experiments at high CO_2 pressures an open-ended mercury manometer is used without any compensating vessel, the omission of which under these conditions leads to no appreciable loss of accuracy. Otherwise the manometer gauges are of simple U-shape without any bottom opening, and after having been cleaned with chromic acid and distilled water and dried with air, are generally filled through one side with de-aerated water. With water in the gauge no contamination of the enzyme by foreign vapour can occur. In a few experiments Clerici's fluid (sp.gr. 4·2) or *iso*-octane (sp.gr. 0·7) has been used in place of water. In experiments with CO_2 pressures of less than 3 cm. of water, the manometer is sloped at a gradient of 1 in 3·5, instead of being vertical, thereby giving about three times greater sensitivity per mm. movement of gauge fluid. With these various devices CO_2 pressures ranging from 0·005 to 0·5 atm. of an atmosphere can readily be used.

The manometers are calibrated by the calculation methods given by Dixon (1934), these being checked by liberation of known volumes of CO_2 from mixtures of HCl and $NaHCO_3$. As an example we quote the details of the calibration for the majority of the experiments. Here the gauge is vertical and filled with water and the total gas pressure in the manometric vessels is initially $\frac{1}{7}$ atm.

Let x = total amount of CO_2, measured in ml. at N.T.P., which is formed by, or disappears as the result of, the chemical reaction in the live vessel,

h = difference in cm. thereby produced between the liquid levels in the two limbs of the manometer,

θ = angle of inclination of U-tube to vertical (usually 0°),

A = area of cross-section of U-tube = 0·024 sq.cm.,

V_G = volume of gas phase in live vessel = 59 ml.,

V_G' = volume of gas phase in compensation vessel = 61 ml.,

$V_L = V_L'$ = volume of liquid in each vessel = 4·2 ml.,

P = total initial pressure in each vessel = 145 cm. water,

p_w = aqueous vapour pressure at 0° = 4 cm. water,

P_0 = the normal pressure = 76 cm. Hg = 1030 cm. water,

α = solubility coefficient of CO_2 in water at 0° = 1·7,

T = absolute temperature = 273°.

With vessels and tubes of these dimensions the following simplified form of Dixon's (1934) general equation (5) gives the calibration factor to less than 1%, namely,

$$x = h\left(\cos\theta + \frac{A(P-p_w)}{2V_G'}\right) \times \left(\frac{V_G}{P_0}\frac{273}{T} + V_L\alpha + \frac{A}{2}\times\frac{273}{T}\times\frac{P-p_w}{P_0}\right), \quad (1)$$

whence $x = h \times 0{\cdot}0677$, i.e. a difference of level of 1 cm. corresponds to a total change of 0·0677 ml. CO_2, of which $0{\cdot}0677 \times 59/(59 + 4{\cdot}2 \times 1{\cdot}7) = 0{\cdot}0604$ ml. represents the change in CO_2 content of the 59 ml. gas phase, and $0{\cdot}0677 \times 4{\cdot}2 \times 1{\cdot}7/(59 + 4{\cdot}2 \times 1{\cdot}7) = 0{\cdot}0073$ ml. represents the change in the amount of CO_2 dissolved in the 4·2 ml. liquid phase, as distinct from that in chemical combination.

Thus the change in molality of dissolved CO_2 corresponding to a difference of gauge level of 1 cm. $= 0{\cdot}0073/4{\cdot}2 \times 22{\cdot}4 = 7{\cdot}76 \times 10^{-5}$ M-HCO_3^- at 0°, whereas the change in the chemically bound CO_2 present in the solution

$$= 0{\cdot}0677/4{\cdot}2 \times 22{\cdot}4 = 7{\cdot}2 \times 10^{-4}\text{ M}. \quad (2)$$

Preparation of buffer solutions. The manometric method has proved suitable for CO_2 output experiments from pH 5·5 to 7·5 and for CO_2 uptake experiments from pH 6·5 to 10·0. The buffer systems which are available over this range are, however, limited, since in many cases one or other of the constituents of the buffer has an inhibitory effect on the enzyme and/or a marked effect on the reactions of CO_2 in water in absence of added enzyme. Experiments to be given elsewhere have, however, shown that the pH range 5·5–9·5 can be satisfactorily covered by the following systems, which, as emphasized below, must be kept free of all but minimal traces of CO_2 (i.e. < 0·0001 M).

(i) Cacodylic acid-sodium cacodylate (pH 5·5–6·5). Solid cacodylic acid and CO_2-free NaOH are dissolved in calculated amounts in CO_2-free twice glass-distilled water, leaving only a small air space in the volumetric flask.

(ii) Phosphate (pH 6·3–7·7). A stock solution of M-KH_2PO_4 is made up by dissolving the calculated amount of the analytical grade salt in CO_2-free twice glass-distilled water. A second stock solution M in total phosphate but containing KH_2PO_4 to K_2HPO_4 in the ratio 1 : 6 is similarly made up and the traces of CO_2 removed by warming the solution to 65° and evacuating at a powerful water-pump, shaking 1 min., letting in air through a dust filter and then repeating the process twenty times. The solution is made up to volume and the ratio of KH_2PO_4 to K_2HPO_4 checked by titration. In many cases $KHCO_3$ has been used in place of K_2HPO_4. Solutions having any desired ratio of KH_2PO_4 to K_2HPO_4 are made by mixing appropriate proportions of the two stock molar solutions.

(iii) Veronal (pH 7·6–8·5). Veronal and its sodium salt are weighed into a glass-stoppered volumetric flask and warm freshly boiled twice glass-distilled water is added so that only a small air space remains. Free veronal is used in place of the HCl customarily used in preparing veronal buffers from the sodium salt, since, below pH 8·0, Cl^- inhibits carbonic anhydrase.

(iv) Dimethylglyoxaline (pH 8·5–9·5). The crystalline hydrochloride of dimethylglyoxaline is hygroscopic. The contents of a sealed tube are therefore dissolved in water to make a solution of about 0·4 M strength, and the molarity found by titration. Portions of this stock solution are diluted with CO_2-free NaOH.

Entry of CO_2 into the solutions after preparation is prevented by keeping them in glass-stoppered vessels with only a small air space. They are used only infrequently and usually only for one group of experiments. Concentrated stock solutions are made whenever solubility permits. The more alkaline solutions are poured quickly into a long burette with a greaseless tap, portions buretted directly into the manometer vessels as required and the upper portions of the liquid column discarded after a few hours. Even in the most alkaline solution used (namely, pH 9·5) the last portion remaining in the burette at the end of the day was found by Van Slyke analysis to contain only 0·00011 M-CO_2.

The reason for such stringency is that appreciable traces of CO_2 would in output experiments raise the substrate concentration and alter the pH by a variable amount, while in uptake experiments the velocity of the back reaction would also be increased to an arbitrary degree. Furthermore, above pH 8·3, the $CO_3^=$ ions increasingly formed would have an inhibitory effect on the enzyme.

To avoid inactivation of the enzyme by metallic impurities, twice glass-distilled water and chemicals of analytical grade purity are used for making up the buffers wherever possible. All glassware used for the enzyme and other solutions is cleaned with chromic acid and then rinsed several times with twice glass-distilled water, contamination with dust and water which had been in contact with metal being sedulously avoided.

Two other solutions besides the stock buffers should also be mentioned:

(v) The bicarbonate solutions used in CO_2 output experiments. Solid $KHCO_3$ is freshly dissolved every few hours in water previously equilibrated with air, transferred to a greaseless burette and withdrawn from the bottom into the manometer vessel. The solution so prepared contains only 1% carbonate, which has but negligible effect on the pH immediately after mixing with the buffer, and is therefore preferred to the stable 5 : 1 bicarbonate : carbonate solution of Hodgson (1936) when used with very dilute

buffer solutions. No appreciable CO_2 is lost from the bicarbonate solution during its brief contact with air while equilibration proceeds.

(vi) CO_2-free NaOH. This is obtained by centrifuging the saturated solution (about 18M-NaOH) in rubber-capped paraffined centrifuge tubes to remove carbonate. The supernatant solution is used directly, a long narrow pipette tip being placed well below the surface, the pipette filled, its outside quickly washed with water, its tip placed below the surface of the water in the volumetric vessel and the NaOH run in. This technique gives the greatest freedom from CO_2 contamination of any we have tried.

Handling of enzyme preparations. Most of our experience has been with the crude 'chloroform enzyme' of Meldrum & Roughton (1933) prepared as follows.

To 10 ml. of washed ox red cells are quickly added 8 ml. of 40% ethanol and 4 ml. of chloroform, the mixture is stirred in a centrifuge tube till the thick paste becomes a thin sludge (about 3 min.) and allowed to stand at least 20 min. before centrifuging. This slight modification in quantities gives a clearer and more colourless solution than the original method. We have also worked with crude laked blood and with the highly purified preparation of Keilin & Mann (1940), but have not yet had the opportunity to study the crystalline preparation of Scott & Fisher (1942).

The activity of the crude chloroform preparation remains constant for at least a year if stored at $-10°$. For testing it should be thawed at a little over 0°, diluted to 1 in 2000 with ice-cold water and then kept in the refrigerator, since the dilute solution, if allowed to warm up to room temperature under certain circumstances, soon loses its activity. The test portion of the diluted enzyme solution (often 0·1 ml.) is measured directly into the buffer solution in the manometric vessel, which is usually already immersed partially in the thermostat. The enzyme solution is now highly diluted, with the result that it has a marked tendency to inactivate during the shaking: this is allowed for in the way described later. In CO_2 uptake experiments there is also a preliminary period of shaking for the purpose of bringing the gases dissolved in the liquids into equilibrium with the gas phase before the CO_2 gas is introduced into the manometric vessel. By reducing this period to 5 sec. the inactivation produced should be inappreciable, whereas the equilibrium between the liquid and gas phases is so nearly reached that the residual exchange when the main shaking starts is mostly balanced by the compensating vessel. The stoppers of the manometric vessels are of rubber but are small and are situated at the top of long necks, so that only very slight contact is made by the enzyme solution with the rubber during the shaking. Some further points under this heading are taken up in other sections of this paper.

THE CALCULATION OF ENZYME ACTIVITY

Changes in the observed activity of the enzyme take place during a manometric determination owing to (i) the inactivation of the enzyme produced by the violent shaking, (ii) the changes during the progress of the experiment in the pH and concentrations of substrate and buffer. To avoid such difficulties we calculate the enzymic activity at a number of instants and extrapolate therefrom to zero time, thus obtaining an activity which is independent of all these changes. Correction is, however, still needed for the limiting effect of diffusion on the observed manometric rates, according to Roughton's (1941) equations, which, as shown below, need modification in certain cases. The requisite elaboration of the diffusion equations is described below (in small type) and may be omitted on a preliminary reading if desired.

Diffusion corrections in CO_2 uptake experiments. Equation (15) of Roughton (1941) is often accurate enough, especially at low CO_2 concentrations, but to define the accuracy precisely we must work out an amendment of his more general equation (29). The latter is not quite exact since it takes no account of the non-enzymic hydration of CO_2 which occurs simultaneously with the enzymic hydration. Roughton & Booth (1938) have shown that the extrapolated value of the non-enzymic hydration rate R_B^0 at zero time is given by

$$R_B^0 = k_u c_L (1 + l[B]) \text{ in mol. } CO_2/\text{L./sec.}, \quad (3)$$

where k_u = velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$ in absence of all catalysts = 0·0021 at 0°,

c_L = concentration of dissolved CO_2 in the bulk of the liquid,

B = the catalytically active constituent of the buffer system,

l = catalytic coefficient of B.

Roughton's equation (28) should therefore be replaced by

R^0 = extrapolated observed rate of CO_2 uptake, at zero time, in presence of enzyme

$$= \frac{D_i A'}{\delta V_L}(c_i - c_L) = \frac{k_e E c_L}{c_L + K_{mu}} + v_u c_L, \quad (4)$$

where D_i = diffusion coefficient of dissolved CO_2,

δ = thickness of the stationary film of liquid at the gas/liquid interface,

A' = area of the stationary film,

V_L = volume of the liquid phase,

c_i = concentration of dissolved CO_2 at the outer surface of the stationary film = αp_i,

α = solubility coefficient of CO_2,

p_i = pressure of CO_2 in gas phase,

K_{mu} = Michaelis constant of enzyme for CO_2 uptake,

k_e = velocity constant for dissociation of CO_2-enzyme complex,

E = concentration of enzyme,

$v_u = k_u(1 + l[B])$.

Equation (14) of Roughton's paper, in the present symbols, reads

R_m = maximum rate of CO_2 uptake, at zero time, which the manometric apparatus can record (i.e. when diffusion is completely the limiting factor)

$$= \frac{D_i A'}{\delta V_L} c_i, \qquad (5)$$

whilst by the Michaelis theory of enzyme action verified elsewhere for carbonic anhydrase (Roughton & Booth, 1946)

R = true rate of CO_2 uptake, at zero time, in presence of enzyme

$$= \frac{k_e E c_i}{c_i + K_{mu}} + v_u c_i. \qquad (6)$$

From (4), (5) and (6) it follows that

$$\frac{R_m}{R^0} = \frac{c_i}{c_i - c_L}, \quad \frac{R_m}{R_m - R^0} = \frac{c_i}{c_L},$$

and

$$\frac{R_m R^0}{R_m - R^0} = \frac{c_i}{c_L} R^0 = \frac{k_e E c_i}{c_L + K_{mu}} + v_u c_i$$
$$= R \frac{c_i + K_{mu}}{c_L + K_{mu}} + v_u c_i \left(1 - \frac{c_i + K_{mu}}{c_L + K_{mu}}\right),$$

whence

$$R = \frac{R_m R^0}{R_m - R^0} \times \frac{c_L + K_{mu}}{c_i + K_{mu}} + v_u c_i \left(1 - \frac{c_L + K_{mu}}{c_i + K_{mu}}\right)$$
$$= \frac{R_m R^0}{R_m - R^0} \left\{1 - \frac{c_i - c_L}{c_i + K_{mu}} \left(1 - \frac{R_m - R^0}{R_m R^0} v_u c_i\right)\right\}$$
$$= \frac{R_m R^0}{R_m - R^0} \left\{1 - \frac{c_i}{c_i + K_{mu}} \frac{R^0}{R_m} \left(1 - \frac{R_m - R^0}{R_m R^0} v_u c_i\right)\right\}. \qquad (7)$$

If $v_u c_i$ is neglected, equation (7) then reduces to equation (29) of Roughton's (1941) paper, namely,

$$R = \frac{R_m R^0}{R_m - R^0} \left(1 - \frac{c_i}{c_i + K_{mu}} \frac{R^0}{R_m}\right).$$

In many cases (see later) sufficient accuracy is attained by the use of the simpler equation

$$R = \frac{R_m R^0}{R_m - R^0}. \qquad (8)$$

Of the five quantities appearing in equation (7):

(*a*) R^0 is determined as described below;

(*b*) v_u, c_i are obtained by the procedure of our previous paper (Roughton & Booth, 1938);

(*c*) R_m is given by Roughton's (1941) method. This is based either on the manometric rate of solution of CO_2 when 4·2 ml. of water are shaken in the usual way with a gas phase containing CO_2, or on the initial rate of CO_2 uptake by buffer solutions containing a high concentration of carbonic anhydrase (the observed manometric rate tends to a maximum as the enzyme concentration is increased). At 0° and with the 4·2 ml. of solution shaken under the usual conditions, both methods agree in giving $D_i A'/V_L = 0{\cdot}63/4{\cdot}2 = 0{\cdot}15$ (see Roughton, 1941, pp. 131, 135), so that $R_m = 0{\cdot}15\ c_i$ mol. CO_2/l./sec.

(*d*) K_{mu} is first obtained roughly from a series of determinations at different substrate concentrations, the effect of diffusion being allowed for by the approximate equation (8). This rough value of K_{mu} is used in equation (7), and the corresponding values of R at the different substrate concentrations then give a more accurate value of K_{mu}. By such process of successive approximation the value of K_{mu} is finally fixed within experimental error.

In the following paper it is shown that at 0°, $K_{mu} = 0{\cdot}009$ over the whole pH range 6·0–9·5. In most of our experiments a standard c_i value of 0·0014 M has been used; R^0/R_m has been kept below 0·2, and since $v_u > 0{\cdot}0021$ it follows that the expression $\frac{c_i}{c_i + K_{mu}} \frac{R^0}{R_m} \left(1 - \frac{R_m - R^0}{R_m R^0} v_u c_i\right)$ is not greater than 0·03, so that equation (8) is accurate enough.* Outside this range the more elaborate equation (7) must be used.

Theoretically a small diffusion correction (of the order of 3%) is also necessary for $R_B{}^0$, namely,

R_B = true rate of CO_2 uptake, at zero time, in absence of enzyme

$$= \frac{R_m R_B{}^0}{R_m - R_B{}^0}. \qquad (9)$$

Finally, we have

R_{eu} = true rate of CO_2 uptake due to enzyme alone $= R - R_B$. (10)

Calculation of R_{eu} in a typical example at pH < 8·0. Fig. 1*A* shows the rate of CO_2 uptake at 0° by a 0·024 M-phosphate buffer solution, pH 7·5, at

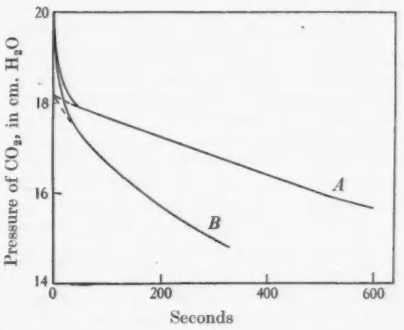

Fig. 1. Rate of CO_2 uptake by 0·024 M-phosphate buffer, pH 7·5, at 0°. *A*, without enzyme; *B*, with enzyme.

the CO_2 pressure used in most of our uptake experiments, namely, 20 cm. H_2O at zero time, whereas Fig. 1*B* shows the effect of adding thereto 14·3 parts per million (p.p.m.) of crude enzyme solution. The curves are as usual diphasic, the first rapid uptake being due to physical solution of CO_2. The slow portion of each curve is extrapolated back to zero time reaching the pressure axis at $p_B^0 = p^0 = 18{\cdot}2$ cm. This is the normal figure, but a range of ± 0·6 cm. H_2O is possible. The times taken for the CO_2 pressure to drop by 3% of the initial value, i.e. by 0·6 cm. H_2O, are then read off for a series of pressure intervals beginning 0·8 cm. below p^0.

* Even in the very fast rate represented by the top point of Fig. 2 in Roughton's (1941) paper, equation (8) is accurate to 8%, since $R^0/R_m = 0{\cdot}6$ and $c_i/(c_i + K_{mu}) = 0{\cdot}14$.

Now a pressure change of 0·6 cm. H_2O corresponds by equation (2) to $0{\cdot}6 \times 7{\cdot}2 \times 10^{-4} = 4{\cdot}32 \times 10^{-4}$ M-HCO_3^- formation, so the reciprocals of these times multiplied by $4{\cdot}32 \times 10^{-4}$ M give the mean values of the over-all enzymic rate, R', and the non-enzymic rate, R'_B, both in mol. HCO_3^-/l./sec. (The calculation of R' and R'_B in the present case is exactly the same as in the detailed example at pH > 8·0 in Table 1.)

Fig. 2 shows the plot of R'/R'_B against t_m, the mean time from the beginning of shaking for each pressure interval, for a duplicate pair of experiments of this kind. The appreciable decay of R'/R'_B with

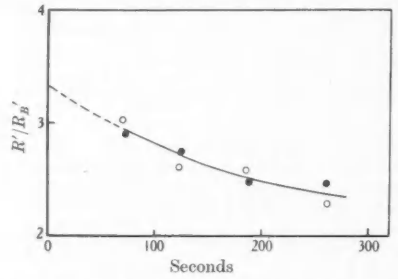

Fig. 2. Curve showing fall in activity of carbonic anhydrase during an experiment at pH <8·0. • First experiment; o duplicate experiment.

time is only to a minor extent due to the lowering of pH through uptake of CO_2 (the enzyme activity is sensitive to pH in this range) and is mainly accounted for by the appreciable rate of inactivation of the enzyme with shaking at pH <8·0. Extrapolation to zero time gives $R^0/R^0_B = 3{\cdot}3$ ($\pm c.$ 10 %). To obtain R^0_B we note that c_L, the extrapolated value of the concentration of dissolved CO_2, at zero time $= 18{\cdot}2 \times 7{\cdot}75 \times 10^{-5} = 1{\cdot}41 \times 10^{-3}$ M, and since in the present phosphate buffer, the concentration of the catalytically active constituent, $HPO_4^=$, = 0·018 M and its l value = 8 (Roughton & Booth, 1938), we have

$$R^0_B = 0{\cdot}0021 \times 1{\cdot}41 \times 10^{-3}\ (1 + 8 \times 0{\cdot}018) = 3{\cdot}39 \times 10^{-6} \text{ mol. } CO_2\text{/l./sec.}$$

Since

$$R_m = 0{\cdot}15 c_i = 0{\cdot}15 \times 1{\cdot}41 \times 10^{-3} = 2{\cdot}11 \times 10^{-4},$$

by equation (8),

$$R_B = \frac{2{\cdot}11 \times 10^{-4} \times 3{\cdot}39 \times 10^{-6}}{2{\cdot}11 \times 10^{-4} - 3{\cdot}39 \times 10^{-6}} = 3{\cdot}45 \times 10^{-6}.$$

Now $$R^0 = 3{\cdot}3 R^0_B = 1{\cdot}14 \times 10^{-5}.$$

In this case equation (8) is also applicable, therefore

$$R = \frac{2{\cdot}11 \times 10^{-4} \times 1{\cdot}14 \times 10^{-5}}{2{\cdot}11 \times 10^{-4} - 1{\cdot}14 \times 10^{-5}} = 1{\cdot}21 \times 10^{-5},$$

whence

$$R_{eu} = R - R_B = 8{\cdot}6 \times 10^{-6} \text{ mol. } CO_2\text{/l./sec.}$$

The value of R_{eu} so computed is usually accurate to ± 10 %, and, as mentioned above, is independent of the inactivation of the enzyme and of the changes in pH, substrate and buffer concentrations during the shaking. In the present example the diffusion corrections only amount in the end to about 7·5 % owing to the low enzyme concentrations used, but at higher values the corrections may become considerable.

All our CO_2 uptake experiments below pH 8·0 have been calculated in this way, pressure intervals equal to 3 % of the total initial CO_2 pressure being generally used for the computations. Pressure changes occurring earlier than 25 sec. from the start of the reaction should, if possible, be avoided for calculation, since the irregularities which are apt to occur just after shaking begins may not have had time to die out. In the instance plotted in Fig. 2 the enzyme has lost about one-quarter of its activity after 200 sec. shaking. In some cases (notably when inhibitory salts are present) we have found losses of a half or even more, and have then plotted log R'/R'_B against t instead. The points so obtained have fallen closely on a straight line, so that extrapolation to zero time can be made with confidence.

Determination of R_{eu} at pH >8·0. In this range the velocity of the back reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ is inappreciable and therefore there should be but little change, during the progress of the experiment, in R'_B/p_m, where p_m = the mean CO_2 pressure for the interval over which R'_B is calculated. This is found to be the case, and it is also found that above pH 8·0 the drift in R'/p_m is usually but slight. More satisfactory extrapolations to zero time are therefore obtained in this range by plotting R'_B/p_m and R'/p_m against time. From such graphs we thus obtain R^0_B and R^0. We then have

$$R^0_B = p^0_B \times (R'_B/p_m \text{ at zero time}) \qquad (11)$$

and

$$R^0 = p^0 \times (R'/p_m \text{ at zero time}). \qquad (12)$$

R_B and R are calculated as above, and R_{eu} is finally obtained. A further advantage of this method is that it avoids (i) any assumption as to the 'l' value of the catalytically active constituent of the buffer, (ii) complications due to the velocity of the reaction $CO_2 + OH^- \rightarrow HCO_3^-$, which becomes significant above pH 8·0.

A typical example of the calculation for 0·038 M-dimethylglyoxaline solution, pH 8·8, 0°, $p_0 = 18{\cdot}6$ cm. H_2O, ± 14·3 p.p.m. crude enzyme solution, is given in Table 1. In this case the diffusion correction finally amounts to about 13 %.

Table 1. *Calculation of enzymic activity at* pH $>8{\cdot}0$ *and* 0°

Absence of enzyme					Presence of enzyme				
p_B (cm. H_2O)	p_m	Time (sec.)	$10^6 R_B'$	$10^7 R_B'/p_m$	p (cm. H_2O)	p_m	Time (sec.)	$10^6 R'$	$10^7 R'/p_m$
17·8	—	96	—	—	17·8	—	32·5	—	—
—	17·5	—	5·93	3·38	—	17·5	—	18·8	10·7
17·2	—	170	—	—	17·2	—	55·9	—	—
—	16·9	—	5·87	3·47	—	16·9	—	18·9	11·2
16·6	—	246	—	—	16·6	—	79·2	—	—
—	16·3	—	5·40	3·31	—	16·3	—	16·7	10·2
16·0	—	327·3	—	—	16·0	—	105·5	—	—
—	15·7	—	5·07	3·23	—	15·7	—	16·4	10·4
15·4	—	414	—	—	15·4	—	132·2	—	—

$R_B^0/p_B^0 = 3{\cdot}56 \times 10^{-7}$. $R^0/p^0 = 11{\cdot}5 \times 10^{-7}$.
$p_B^0 = 18{\cdot}6$ cm. $p^0 = 18{\cdot}6$.
$R_B^0 = 0{\cdot}66 \times 10^{-5}$ mol. CO_2/l./sec. $R^0 = 2{\cdot}14 \times 10^{-5}$ mol. CO_2/l./sec.
$R_B = 0{\cdot}68 \times 10^{-5}$ mol. CO_2/l./sec. $R = 2{\cdot}36 \times 10^{-5}$ mol. CO_2/l./sec.
$R_{eu} = 1{\cdot}68 \times 10^{-5}$ mol. CO_2/l./sec.

Corrected equations for the effect of diffusion in CO_2 *output experiments*

Equation (30) of Roughton's (1941) paper is also at fault through neglect of the velocity of the non-enzymic reaction, the effect of which is quite significant at pH 6·0 and below. Taking this into account we have, on the basis of the treatment given on p. 136 of his paper, at zero time,

$$\frac{DA'}{\delta V_L} c_L = \frac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L - \frac{k_e E c_L}{c_L + K_{mu}} - v_u c_L = R^0, \quad (13)$$

where x_L = concentration of H_2CO_3 in the bulk of the liquid phase,

K_{mo} = Michaelis constant for CO_2 output,

k_e = velocity constant for the dissociation of the enzyme-H_2CO_3 complex = velocity constant for the dissociation of the enzyme-CO_2 complex (Roughton & Booth, 1946),

$v_0 = k_0(1 + l[B])$,

k_0 = velocity constant of the $H_2CO_3 \rightarrow CO_2 + H_2O$ reaction in absence of catalyst,

K = equilibrium constant of the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O = k_0/k_u = v_0/v_u$.

As shown elsewhere (Roughton & Booth, 1946) $K_{mu} = KK_{mo}$. Therefore equation (13) reduces to

$$R^0 = \frac{DA'}{\delta V_L} c_L = \frac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L - \frac{k_e E c_L}{c_L + KK_{mo}} - \frac{v_0}{K} c_L. \quad (14)$$

As $t \rightarrow 0$, we have

$$R_B = v_0 x_L, \quad (15)$$

$$R = \frac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L, \quad (16)$$

R_{mo} = maximum output velocity rate at very high enzyme concentration

$$= \frac{DA'}{\delta V_L} x_L K, \quad (17)$$

for in this case the CO_2 and H_2CO_3 are always in equilibrium in the body of the liquid and therefore $c_L = x_L K$.

From (14) and (17),

$$\frac{R^0}{R_{mo}} = \frac{c_L}{x_L K} \quad \text{or} \quad c_L = x_L K \frac{R^0}{R_{mo}}.$$

From (14) and (16),

$$\frac{R}{R^0} = \frac{\dfrac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L}{k_e E \left(\dfrac{x_L}{x_L + K_{mo}} - \dfrac{c_L}{c_L + KK_{mo}} \right) + v_0 \left(x_L - \dfrac{c_L}{K} \right)}$$

$$= \frac{\dfrac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L}{k_e E \left(\dfrac{x_L}{x_L + K_{mo}} - \dfrac{x_L K R^0 / R_{mo}}{x_L K R^0 / R_{mo} + KK_{mo}} \right) + v_0 (x_L - x_L R^0 / R_{mo})}$$

$$= \frac{\dfrac{k_e E}{x_L + K_{mo}} + v_0}{\dfrac{R_{mo} - R^0}{R_{mo}} \left\{ \dfrac{k_e E K K_{mo}}{(x_L + K_{mo})(x_L K R^0 / R_{mo} + KK_{mo})} + v_0 \right\}},$$

whence
$$R=\frac{R_{mo}R^0}{R_{mo}-R^0}\left\{\frac{k_eE(x_LKR^0/R_{mo}+KK_{mo})+v_0(x_L+K_{mo})\,(x_LKR^0/R_{mo}+KK_{mo})}{k_eEKK_{mo}+v_0(x_L+K_{mo})\,(x_LKR^0/R_{mo}+KK_{mo})}\right\}$$
$$=\frac{R_{mo}R^0}{R_{mo}-R^0}\left\{1+\frac{x_LR^0/R_{mo}}{K_{mo}+\dfrac{v_0}{k_eE}(x_L+K_{mo})\,(x_LR^0/R_{mo}+K_{mo})}\right\}$$
$$=\frac{R_{mo}R^0}{R_{mo}-R^0}\left\{1+\frac{x_LR^0/R_{mo}}{K_{mo}+\dfrac{R_B(x_LR^0/R_{mo}+K_{mo})}{R-R_B}}\right\}. \qquad (18)$$

Since, as may be shown from equations (15) and (16),

$$\frac{v_0}{k_eE}(x_L+K_{mo})=\frac{R_B}{R-R_B}.$$

If v_0 is neglected, equation (18) reduces to equation (30) of Roughton's (1941) paper, namely,

$$R=\frac{R_{mo}R^0}{R_{mo}-R^0}\left(1+\frac{x_LR^0}{K_{mo}R_m}\right).$$

The value of R is calculated from equation (18) by successive approximation, $R_B/(R-R_B)$ first being placed $=R_B^0/(R^0-R_B^0)$, which is given directly by extrapolation from the experiments. From the value of R so obtained, a new value of $R_B/(R-R_B)$ is derived, inserted in equation (18), R recalculated and so on. Even in the most unfavourable case, three such successive approximations have proved enough to fix R to within 2%: often a single approximation process is sufficient. We also have

$$R_B=\frac{R_{mo}R_B^0}{R_{mo}-R_B^0}, \qquad (19)$$

whence $R_{co}=R-R_B$ is obtained.

Determination of R_{mo}. Roughton (1941) has shown theoretically, and confirmed by experiment, that

$$R_{mo}=\text{constant}\times\frac{b}{1+K_1'/f_{HCO_3}a_H}, \qquad (20)$$

where b = total CO_2 in all forms at zero time
$$=[CO_2]+[H_2CO_3]+[HCO_3^-],$$
K_1' = apparent first ionization constant of carbonic acid
$$=f_{HCO_3}a_H[HCO_3^-]/([CO_2]+[H_2CO_3])$$
$$=2{\cdot}61\times10^{-7}\text{ at }0^\circ\text{ (Shedlovsky \& MacInnes, 1935)},$$
f_{HCO_3} = activity coefficient of bicarbonate ion,
a_H = hydrogen-ion activity of solution.

The value of this constant, R_{mo}, according to Roughton's treatment, is the same as that of the constant in equation (5) for R_m, namely, 0·15 for the particular apparatus and shaking conditions used by us.

Determination of $f_{HCO_3}a_H$. In cacodylate buffer solutions we have

$$a_H=K_C[HCac]/f_C[Cac^-], \qquad (21)$$

where K_C = ionization constant of cacodylic acid,
f_C = activity coefficient of cacodylate ion.

Therefore

$$f_{HCO_3}a_H=K_C[HCac]f_{HCO_3}/f_C[Cac^-]. \qquad (22)$$

Now because HCO_3^- and Cac^- are both univalent anions, it follows from the Debye-Huckel theory of dilute solutions that

$$f_{HCO_3}=f_C=\text{antilog}\,(-0{\cdot}5\sqrt{\mu}), \qquad (23)$$

where μ = ionic strength of the solution. This relationship is experimentally confirmed by the electrometric determinations of Hastings & Sendroy (1925) on CO_2-H_2CO_3 mixtures, and of Morton (1928) on cacodylate buffer solutions. Therefore

$$f_{HCO_3}a_H=K_C[HCac]/[Cac^-]. \qquad (24)$$

The numerical value of K_C at 0° is taken as $10^{-6\cdot29}$. (See note at the end of this paper.)

Most of our CO_2 output experiments have been done in cacodylate and phosphate buffer solutions. In the latter the activity corrections are complex owing to the presence of bivalent $HPO_4^=$ ions and no such simple equation as (24) can be used. Instead, we determine the value of $f_{HCO_3}a_H$, at zero time, closely enough by comparing the observed rate of CO_2 output, R_p^0, as $t\to0$ in the phosphate buffer experiment with the observed rate of CO_2 output, R_C^0, in a corresponding cacodylate buffer experiment. We then have

$$\frac{R_p^0}{R_C^0}=\frac{(f_{HCO_3}a_H[HCO_3^-])\text{ in phosphate experiment}\times(1+l_p[HPO_4^=])}{(f_{HCO_3}a_H[HCO_3^-])\text{ in cacodylate experiment}\times(1+l_C[Cac^-])}, \qquad (25)$$

$l_p=8{\cdot}0$, $l_C=9{\cdot}0$ and, all the other quantitites in equation (25) being known, $f_{HCO_3}a_H$ for the phosphate solution is then calculable. A similar procedure has been used in the few experiments with other buffer solutions.

Determination of K_{mo}/x_L. This is the remaining quantity still required before equation (18) can be applied. We have

$$f_{HCO_3}a_H[HCO_3^-] = K_1[H_2CO_3] = K_1'([CO_2]+[H_2CO_3]), \quad (26)$$

where K_1 = true first ionization constant of carbonic acid. Therefore

$$K_1 = K_1'(1+K) = KK_1', \quad (27)$$

since K is of the order of 900 at 0° (see Roughton, 1941), and since

$$K = k_0/k_u,$$

$$k_0/K_1 = k_u/K_1'. \quad (28)$$

So $x_L = [H_2CO_3] = f_{HCO_3}a_H[HCO_3^-]/K_1$
$= f_{HCO_3}a_H[HCO_3^-]/KK_1'$.

Now $K_{mo} = K_{mu}/K$. Therefore

$$K_{mo}/x_L = K_{mu}K_1'/f_{HCO_3}a_H[HCO_3^-]$$
$$= 9\times10^{-3}\times2{\cdot}61\times10^{-7}/f_{HCO_3}a_H[HCO_3^-]$$
$$= 2{\cdot}35\times10^{-9}/f_{HCO_3}a_H[HCO_3^-] \text{ at } 0°. \quad (29)$$

Calculation of R_{eo} in cacodylate experiments. As an example we shall describe the evaluation of R_{eo} in case of CO_2 output at 0° from a solution consisting initially of 0·0025 M-$NaHCO_3$, 0·0192 M-HCac, 0·0048 M-NaCac + 57 p.p.m. of crude enzyme. The total CO_2 evolved gave rise to a gauge level difference of 10 cm. and Table 2 shows the respective times taken over four pressure intervals of 1 cm. starting after a 2 cm. difference of level has been produced. R'/R_B' is thence plotted against the mean time corresponding to each interval just as in the CO_2 uptake procedure at pH < 8·0, and extrapolation to zero time gives $R^0/R_B^0 = 2{\cdot}6$.

We have by equation (24), at zero time,

$$f_{HCO_3}a_H = 10^{-6\cdot29}\times0{\cdot}0192/0{\cdot}0048 = 2{\cdot}1\times10^{-6}.$$

Therefore by equation (20),

$$R_{mo} = (0{\cdot}15\times0{\cdot}0025)/(1+2{\cdot}6\times10^{-7}/2{\cdot}1\times10^{-6})$$
$$= 3{\cdot}3\times10^{-4} \text{ mol. } CO_2/\text{l./sec.}$$

Table 2. *Calculation of R_{eo} in cacodylate buffer solution*

Presence of enzyme		Absence of enzyme		
p_B (cm H_2O)	Time (sec.)	p_B (cm. H_2O)	Time (sec.)	R'/R_B'
2	10·7	2	21·4	2·74
3	14·2	3	31·0	2·72
4	18·2	4	41·9	2·91
5	23·0	5	55·6	2·96
6	28·4	6	73·2	

$\frac{R'}{R_B'} = \frac{\text{time to traverse pressure interval in absence of enzyme}}{\text{time to traverse same pressure interval in presence of enzyme}}$.

From Roughton & Booth's (1938) data,

$$R_B^0 = k_0(1+l_C[Cac^-])[HCO_3^-]f_{HCO_3}a_H/K_1$$
$$= k_u(1+l_C[Cac^-])[HCO_3^-]f_{HCO_3}a_H/K_1$$
$$= 0{\cdot}0021(1+9\times0{\cdot}0048)\times0{\cdot}0025\times2{\cdot}1\times10^{-6}/2{\cdot}6\times10^{-7}$$
$$= 4{\cdot}43\times10^{-5}.$$

Therefore

$$R^0 = 2{\cdot}6\times4{\cdot}43\times10^{-5} = 1{\cdot}15\times10^{-4} = 0{\cdot}35R_{mo},$$

$$R_B^0/(R^0-R_B^0) = 0{\cdot}628,$$

and by equation (29),

$$K_{mo}/x_L = 2{\cdot}35\times10^{-9}/(2{\cdot}1\times10^{-6}\times0{\cdot}0025) = 0{\cdot}45.$$

Substituting these values in equation (18) we get

$$R = 2{\cdot}42\times10^{-4}.$$

Since

$$R_B = (3{\cdot}3\times10^{-4}\times4{\cdot}43\times10^{-5})/(3{\cdot}3\times10^{-4}-4{\cdot}43\times10^{-5}) = 5{\cdot}12\times10^{-5},$$

$R_B/(R-R_B) = 0{\cdot}268$ to a first approximation. Substituting in equation (18) we get $R = 2{\cdot}69\times10^{-4}$ to a second approximation. A third approximation gives $R = 2{\cdot}73\times10^{-4}$. Therefore

$$R_{eo} = 2{\cdot}73\times10^{-4}-5{\cdot}12\times10^{-5}$$
$$= 2{\cdot}22\times10^{-4} \text{ mol. } CO_2/\text{l./sec.}$$

If no correction for diffusion had been applied, the enzymic activity would have been taken as equal to $(R^0-R_B^0)$, i.e. to $0{\cdot}71\times10^{-4}$, which is only one-third of the corrected value for R_{eo}. The influence of diffusion in this instance is thus very large, owing to the high speed of the non-enzymic as well as of the enzymic reaction at the relatively acid pH of 5·6; larger corrections than this seem to be of questionable validity, and we have therefore felt it unsafe to push the manometric method to more acid pH than 5·6. As the pH is raised the diffusion correction rapidly decreases: thus in cacodylate buffer at pH 6·4 and with the same enzyme concentration as that in the above experiment the diffusion correction is only 25%.

The theoretical treatment and experiments quoted by Roughton (1941, pp. 138–40) indicate that in output experiments the initial irregularities after shaking begins should disappear in about 10 sec., and hence times as early as this have been utilized in Table 2. The reason that the initial irregular period is so much shorter than in CO_2 uptake experiments, wherein a minimum of 25 sec. should be allowed before readings are used, is that in the latter case a much greater formation of dissolved CO_2 occurs in the initial period just after shaking begins.

Exactly the same method of calculation has been used in all our output experiments. The value of R'/R_B' is often found to rise appreciably with time

during the progress of the experiment, the inactivation brought about by the shaking being more than compensated for by the increase in relative activity of the enzyme as the solution becomes more alkaline through loss of CO_2 and as the substrate concentration decreases (see Discussion).

DISCUSSION

The above methods of computing carbonic anhydrase activity have been cumbrous to describe, but after some practice become rapid in actual use. Of the necessity of using them properly pointed instances are given in the following paper (Roughton & Booth, 1946); e.g. (i) Our first experiments on the pH activity curve of the enzyme were done before the diffusion corrections had been completely worked out: when these were correctly applied to the data, a curve of quite different shape was obtained. In our first calculation we had underestimated the size of the corrections at acid pH. (ii) In an early set of experiments on the activity of a crude chloroform enzyme preparation kept at 0° we found an apparent rise in enzymic activity during storage: in these we have, however, calculated the activity of the enzyme from its magnifying effect on the rate of CO_2 exchange during the second quarter of the manometric process, as was done originally by Meldrum & Roughton (1933), but we had made no allowance for the inactivation of the enzyme during shaking. When, however, the activity was extrapolated to zero time as above, it was found to be the same within experimental error; the older preparations, therefore, had not increased in absolute activity, but had apparently suffered some change which depressed the rate of inactivation during the manometric shaking.

The activity of the enzyme in a given buffer solution should theoretically be proportional to the amount added: we have sometimes found, especially in our earlier work before full cleanliness precautions had been adopted (see Fig. 1 of Roughton & Booth, 1946), that with small amounts of enzyme the observed effect is proportionally much less than with large amounts. This is probably due to traces of metals, or other inactivating impurities, in the apparatus or solutions, the first amounts of added enzyme combining with and removing the impurity thus enabling further enzyme to exert its full activity. Frequent controls should therefore be made as to proportionality between activity and amount of enzyme added.

In the following paper we show that the activity of the enzyme, as measured manometrically, agrees fairly closely with that measured colorimetrically in an all-liquid system, wherein no inactivation, adsorption or other effects at the gas/liquid interface could have occurred. This result gives some reply to the most obvious criticism that can be levelled against the manometric method of measuring carbonic anhydrase activity. The other methods of measuring the enzyme activity are also more appropriately discussed in that paper (Roughton & Booth, 1946) in light of the new data given there on the effect of various ions and of substrate concentration on the activity of the enzyme.

Note on the ionization constant of cacodylic acid at 0°

Faurholt's (1924) value for the ionization constant of cacodylic acid (K_C) at 0° was based on the equation

$$\frac{K_C}{K_1} = \frac{[\text{HCac}]\,[\text{HCO}_3^-]}{[\text{Cac}^-]\,[\text{CO}_2]},$$

where the symbols in brackets represent the concentrations of the substances at equilibrium, when known amounts of CO_2 or bicarbonate are added to cacodylate buffer solutions. Faurholt measured the value of $[CO_2]$ at equilibrium by his carbamino method, and deduced the concentrations of the remaining substances from their initial values and the found value of $[CO_2]$. A small error in $[CO_2]$ would therefore give rise to a several times larger error in K_C/K_1'. Faurholt's actual value for K_C/K_1' was $2{\cdot}34 \pm 0{\cdot}2$, and assuming Kendall's value for K_1' of $2{\cdot}24 \times 10^{-7}$ at 0° he thence calculated $K_C = 10^{-6{\cdot}29}$.

In manometric experiments on the rate of CO_2 output at 0° from a solution containing 0·016 M-HCac, 0·024 M-NaCac, 0·005 M-$NaHCO_3$, we found

$$\frac{d[\text{CO}_2]}{dt} = 3{\cdot}40 \times 10^{-3}[\text{HCO}_3^-].$$

Theoretically

$$\frac{d[\text{CO}_2]}{dt} = \frac{k_0 f_{\text{HCO}_3} a_{\text{H}} [\text{HCO}_3^-]}{K_1}(1 + l[\text{Cac}^-])$$

$$= k_u \frac{K_C\,[\text{HCac}]}{K_1'\,[\text{Cac}^-]}\,[\text{HCO}_3^-]\,(1 + l\,[\text{Cac}^-]).$$

Since $k_u = 0{\cdot}0021$, $[\text{HCac}]/[\text{Cac}^-] = 0{\cdot}67$, $l = 9$, $[\text{Cac}^-] = 0{\cdot}024$, we get $K_C/K_1' = 1{\cdot}98$.

Taking the more recent and better established value of Shedlovsky & MacInnes (1935) for K_1' of $2{\cdot}61 \times 10^{-7}$ at 0°, we find $K_C = 5{\cdot}17 \times 10^{-7} = 10^{-6{\cdot}29}$. This value by coincidence agrees exactly with that of Faurholt. We believe it to be correct to within ±10%, and have used it in all CO_2 output calculations. At 30° Morton (1928) reports that $K_C = 5{\cdot}66 \times 10^{-7} = 10^{-6{\cdot}25}$.

SUMMARY

1. Improvements are described in the technique and calibration of the manometric apparatus for measuring carbonic anhydrase activity. For accurate results special precautions are needed in the preparation of the buffer solutions and for the handling of the enzyme preparations.

2. To study the kinetics of carbonic anhydrase under varied conditions it is necessary to express the activity of the enzyme, not in some arbitrary units, but in terms of the actual extra rate, due to the presence of the enzyme, of CO_2 uptake or output in mol. CO_2/l. solution/sec. Directions are given for

calculating the extra rate in this unit at a series of instants during the course of the manometric experiment. These extra rates are then extrapolated to zero time so as to eliminate changes in the activity of the enzyme during the actual experiment owing to (i) progressive alterations in pH, substrate and buffer concentrations as CO_2 is taken up or evolved, (ii) destruction brought about by the violent shaking. The extrapolated rates are finally corrected for the limiting effect of diffusion by means of extensions of the equations previously developed by Roughton, on the basis of the application of the stationary film theory to gas-enzymic reactions.

3. Detailed examples of the computation of enzymic activity are given for CO_2 uptake experiments both below and above pH 8·0 and also for CO_2 output experiments.

4. Applications of the methods to the study of the kinetics of carbonic anhydrase under varied conditions are given in the following paper.

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The Effect of Substrate Concentration, pH and other Factors upon the Activity of Carbonic Anhydrase

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This paper presents data obtained between 1936 and 1940 on the effects of substrate concentration, pH and salts on the activity of carbonic anhydrase at 0°. A brief summary of the more important findings has already been given (Roughton, 1943). Completion of the work has been much delayed, in part by difficulties in working out the results, but in the main by preoccupation of both authors with research related to the war. During the interim, important advances have been made in other directions, e.g. the demonstration of zinc as the active metal of the enzyme (Keilin & Mann, 1940), the crystallization of the enzyme by Scott & Fisher (1942), the claim of the existence of activators by Leiner (1940), and the discovery of the inhibitory effect of sulphanilamide (Mann & Keilin, 1940). Most of our data, however, seem still to retain their value, and after presenting them we shall discuss their significance in the light of the more recent work.

The topics to be dealt with are routine ones in the study of the kinetics of an enzyme, and it might well be asked why such results were not published within a year or so of the discovery of carbonic anhydrase by Meldrum & Roughton (1932). The answer is that in the case of this enzyme a number of unusual difficulties were encountered which had first to be resolved. As regards substrate concentration Meldrum & Roughton's (1933) preliminary observations at 15° suggested that the Michaelis constant in the case of CO_2 uptake by the enzyme might be so high that for the enzyme to be largely saturated with substrate it would be necessary to work at CO_2 pressures of several atmospheres. At 0°, however, the Michaelis constant is much lowered and adequate data can be obtained with CO_2 pressures ranging merely from 0·01 to 1·0 atm., which are well within the scope of ordinary manometric technique. As regards the effect of pH and salts the main difficulty has been that carbonic anhydrase,

unlike most other enzymes, catalyzes reactions which not only proceed at appreciable rates in absence of the enzyme but are also catalyzed by non-enzymic solutes, especially the more electronegative constituent of many buffer systems. Thus at pH below 8·0, the true overall rate of hydration of CO_2 is, according to Roughton & Booth (1938), Kiese & Hastings (1940*a*, *b*), and Roughton (1943), given by

$$-\frac{d[CO_2]}{dt} = \{k_u[CO_2] - k_0[H_2CO_3]\}\{1 + l[B]\}, \quad (a)$$

where $k_u = 0{\cdot}0021$ at 0°,

$k_0 = 2{\cdot}0$ at 0°,

B = catalytically active constituent, i.e. $HPO_4^=$ in the case of phosphate buffer,

l = a catalytic coefficient of value, e.g. 8 for $HPO_4^=$, *c.* 1700 for $SeO_3^=$, *c.* 10,000 for $H_2AsO_3^-$, *c.* 30,000 for BrO^-.

Above pH 8·0 the rate of hydration begins to increase independently of the buffer concentration. The extra rate is proportional to the OH^- concentration, being given by

$$-\frac{d[CO_2]}{dt} = k'_u[CO_2][OH^-] - k'_0[HCO_3^-], \quad (b)$$

where k'_u is of the order of 1000 at 0°. Equation (*b*) may be equally well explained either by a direct reaction, $CO_2 + OH^- \rightarrow HCO_3^-$, or as due to a catalysis of the $CO_2 + H_2O \rightarrow H_2CO_3$ reaction by OH^- ions, the 'l' value of the latter being outstandingly large, namely, about 500,000. At present there is no reason to prefer one mechanism to the other, but on either view it seems unlikely that so intrinsically rapid a reaction would itself be further catalyzed by other substances. In our previous paper (1938) we did indeed show that phosphate, cacodylate and borate, though they markedly catalyze the $CO_2 + H_2O \rightarrow H_2CO_3$ reaction, were all without effect on the process corresponding to equation (*b*). The same would probably have been found of the other buffers of our series had these too been investigated in similar detail.

Direct tests in the case of carbonic anhydrase are more elusive, though Kiese (1941) has brought forward definite theoretical and experimental arguments that process (*b*) is not catalyzed by the enzyme and this viewpoint will be adopted in the present paper. Even, however, with this simplification, the task of working out the catalytic action of carbonic anhydrase in buffer solutions is still a complicated one. Not only have there to be considered (i) the effect of pH upon the catalysis by the enzyme of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ but also (ii) the possible effect of the enzyme upon the catalysis of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ by the more electronegative constituents of the buffer when such catalysis is but slight (as opposed to the large catalysis which OH^- may produce, as just mentioned), (iii) possible inhibitory or activating effects of the buffer upon the enzyme. Of these, (ii) and (iii) have been investigated by varying the total buffer concentration at constant enzyme and substrate concentrations. With the buffers used in the present work, over the range pH 5·5–10·0, the effects seem to be either negligible at buffer concentrations below 0·05 M, or small enough to be allowed for by extrapolation to zero buffer concentration. Several buffers of lower pK and salts of strong acids, however, show marked depressions of enzymic activity even at concentrations of 0·01 M and are therefore unsuitable.

EXPERIMENTAL METHODS

Apart from a few control experiments, in which the activity of the enzyme was measured by an all liquid indicator method (see later), all the results to be reported below were obtained at 0° by the boat-manometric technique described by Roughton & Booth (1938, 1946).

The buffer solutions were prepared and kept as free of CO_2 as possible, in the way described by Roughton & Booth (1946). The main buffers used were cacodylate, phosphate, veronal and dimethylglyoxaline, these covering the pH range to be studied, namely, pH 5·5–9·5. A few supplementary experiments were also done with pyrophosphate, allantoin and succinimide buffer solutions, prepared and treated in similar manner.

Most of our experiments have been made with the crude chloroform preparation of Meldrum & Roughton (1933), but some with solutions of lyzed red cells and a few data with the highly purified preparations of Mann & Keilin (1940). The crystalline preparation of Scott & Fisher (1942) was not available at the time of our work. The enzyme solutions were prepared and handled with the precautions described elsewhere (Roughton & Booth, 1946), and under these conditions the crude chloroform preparation used for the bulk of the experiments remained constant in activity (within experimental error) for at least a year, as has been the experience also of more recent observers. So far, no obvious differences have been found in the kinetics of the enzyme at various grades of purity save that the tendency to inactivation during manipulation and manometric shaking is increased by each stage of purification, especially the later ones.

THE CALCULATION OF THE ENZYMIC ACTIVITY

The activities of the enzyme are, throughout this paper, expressed as the additional rate of CO_2 uptake or output, when enzyme is added, over and above the rate of CO_2 uptake by or output from the same buffer solution in absence of enzyme. They are given in mol. CO_2/l./sec., the calculations being

made by the methods already described in detail (Roughton & Booth, 1946): these allow not only for the changes in activity of the enzyme during the actual manometric shaking but also for the limiting effect of diffusion between the gas and liquid phases. In these respects, especially the latter, the results are superior to those of most previous manometric determinations, wherein furthermore the activity has often been expressed as a *magnification* of, rather than an *addition* to, the non-enzymic rate. The addition method of expressing activity is, however, indispensable when studying the effect of substrate concentration, and is likewise much more serviceable in disentangling the effects of pH and buffer concentration. Values given by it are, indeed, independent of any assumption of the physico-chemical mechanism of the enzyme reactions. The magnification method is useful in comparing the number of enzyme units in various preparations, but cannot cope properly with most of the questions dealt with in this paper.

EFFECT OF ENZYME CONCENTRATION

Previous workers have shown that the extra rate of reaction in presence of carbonic anhydrase is proportional to the concentration of the enzyme. Thus, Meldrum & Roughton (1933) found it so for blood solutions in M/10 phosphate buffer, over the narrow range in which corrections for the effect of diffusion are small, whilst, with allowance for the latter, Roughton (1941, Fig. 2) found that the relation between crude chloroform enzyme concentration and extra rate was linear to within $\pm 5\%$ over a 40-fold range when measured in M/40 phosphate, pH 7·5, 0°. We have made altogether 12 tests with blood solution and crude chloroform preparation as source of enzyme over the range pH 5·5–9·5 and with the whole available set of buffers. The enzyme concentration was varied over the useful range of the apparatus. In almost every case a linear relationship held good to $\pm 10\%$, the average divergency being only 4%, which is well within the limits of error of the activity measurements.

In about half of our preliminary experiments on the highly purified preparation of Mann & Keilin we found, however, an inflected curve like that shown in Fig. 1*B*. In this it is seen that low concentrations of enzyme produce only slight effect but that after a certain amount has been added the effect of further additions is practically linear. Fig. 1*B* represents the results obtained on a crude chloroform preparation in M/40 phosphate, pH 7·5, at an early stage of the work before all the necessary technical precautions (Roughton & Booth, 1946) had been fully mastered. For example, the solutions were prepared in water distilled from a metal, instead of an all-glass, still. Six weeks later, when the technique had been fortified, we obtained with the same enzyme solution the linear relationship shown in Fig. 1*A*. It will be noted, however, that the steep linear portion of Fig. 1*B* is parallel to the straight line of Fig. 1*A*, so that if the activity had been calculated from the slope of the steep linear portion of Fig. 1*B* a correct result would have been

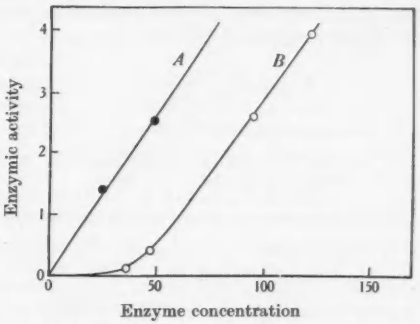

Fig. 1. Activity-enzyme concentration curve for carbonic anhydrase. *A*, the usual type of curve; *B*, occasional type.

obtained. This has been the basis of our activity calculation in those experiments with the highly purified preparation of Mann & Keilin, in which, in spite of all precautions, we obtained a curve of the type of Fig. 1*B*. As to the cause for such curves, one possibility is that in the experiment of Fig. 1*B*, there was some definite amount of metal or other enzyme poison present in the solutions and/or apparatus, and that an equivalent amount of enzyme was inactivated by combination with such impurity. After the latter had been removed thereby, further amounts of enzyme would have been free to exert their full proportional effects. In highly purified enzyme preparations the total amount of protein is so low that this phenomenon becomes increasingly difficult to guard against: another possibility is the adsorption of a definite amount of the enzyme at the solid/liquid or gas/liquid interfaces in the manometric vessel, these surfaces being preferentially occupied by other substances in the case of less pure enzyme preparations. Scott & Fisher (1942) and Scott & Mendive (1941*a*, *b*) have, indeed, found it necessary to add small quantities of peptone when measuring the activity of highly purified or crystalline preparations of the enzyme.

SUBSTRATE CONCENTRATION AND THE MICHAELIS CONSTANT

According to the Michaelis theory of enzyme reaction, the extra rate of CO_2 uptake in presence of the enzyme should be given by

$$R_{eu} = \frac{k_{eu} c E}{c + K_{mu}}, \qquad (1)$$

where c = concentration of dissolved CO_2,

E = concentration of the enzyme,

k_{eu} = velocity constant for dissociation of the enzyme complex $E.CO_2$,

K_{mu} = Michaelis constant for CO_2 uptake.

Similarly the extra rate of CO_2 output in presence of the enzyme should be given by

$$R_{eo} = \frac{k_{eo} x E}{x + K_{mo}}, \qquad (2)$$

where x = concentration of H_2CO_3,

k_{eo} = velocity constant for dissociation of the enzyme complex $E.H_2CO_3$,

K_{mo} = Michaelis constant for CO_2 output.

Let K = equilibrium constant of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$

= about 900 at 0° (Roughton, 1943),

c_∞, x_∞ = the respective concentrations of CO_2 and H_2CO_3 at equilibrium.

Then $c_\infty = K x_\infty$.

Since at equilibrium the rates of CO_2 uptake and CO_2 output must both be equally catalyzed by the enzyme

$$\frac{k_{eu} E c_\infty}{c_\infty + K_{mu}} = \frac{k_{eo} E x_\infty}{x_\infty + K_{mo}} = \frac{k_{eo} E c_\infty / K}{c_\infty / K + K_{mo}} = \frac{k_{eo} E c_\infty}{c_\infty + K K_{mo}}. \qquad (3)$$

Equation (3) is true whatever value of c_∞ be selected. This can only be so if

$$k_{eu} = k_{eo} = k_e \qquad (4)$$

and

$$K_{mu} = K K_{mo}. \qquad (5)$$

Equations (4) and (5) have already been made use of for calculating enzymic activity from CO_2 output experiments (Roughton & Booth, 1946).

Determination of K_{mu}. Equation (1) has been tested at three to four CO_2 concentrations, ranging from 0·001 to 0·02 M, (i) at pH 7·5 (M/40 phosphate), (ii) at pH 8·0 (M/24 veronal), (iii) at pH 9·3 (M/15 dimethylglyoxaline), in the case of the crude chloroform enzyme solution, the final dilution of the latter being 14·3 parts per million (p.p.m.). For the low CO_2 pressures the manometer gauge was filled as usual with water, but for the higher ones an open-ended mercury gauge was used, or Clerici fluid was placed in the U-tube gauge. In correcting for the limiting effect of diffusion, the simple equation $R = R_m R^0/(R_m - R^0)$ was generally adequate, but in a few cases the more detailed method of approximation described in our adjoining paper (1946) was used.

From equations (1) and (4) it follows that

$$\frac{c}{R_{eu}} = \frac{c + K_{mu}}{k_e E}. \qquad (6)$$

The circles in Fig. 2 show the plot of c/R_{eu} against c in each of the above three cases. The points almost all fall on straight lines within experimental error as equation (6) requires. K_{mu} is given by the distance from the origin at which each line intercepts

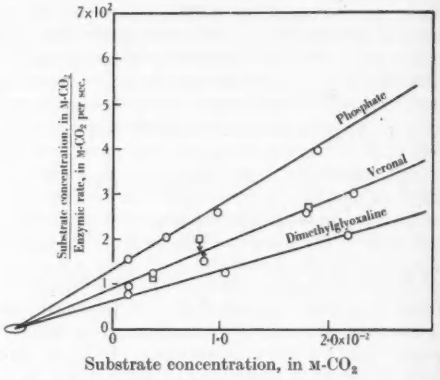

Fig. 2. Effect of substrate concentration on activity of carbonic anhydrase. o = chloroform enzyme solution, 14·3 p.p.m. □ = Red cell solution, 7·2 p.p.m.

the c-axis and $k_e E$ by the cotangent of the angle made with the c-axis. It is clear from Fig. 2 that k_e varies markedly with pH, but that the value of K_{mu}, namely 0·009 ± 0·001 M at 0°, is roughly constant. Kiese & Hasting's finding (1940*b*) that the pH activity curve of carbonic anhydrase is the same when measured at two different CO_2 pressures (100 and 230 mm. Hg) over the more extended range pH 6·1–10·0 leads to the same conclusion. We did not attempt to determine K_{mu} directly below pH 7·0, since the total CO_2 uptake at low CO_2 pressures then becomes too small for accurate measurement.

From equations (2), (4) and (5) of this paper and equation (29) of the adjoining paper it follows that

$$R_{eo} = \frac{k_e E}{1 + K_{mo}/x} = \frac{k_e E}{1 + K_{mu} K_1'/f_{HCO_3} a_H [HCO_3^-]} = \frac{k_e E [HCO_3^-]}{[HCO_3^-] + K_{mu} K_1'/f_{HCO_3} a_H}, \qquad (7)$$

whence

$$\frac{[HCO_3^-]}{R_{eo}} = \frac{[HCO_3^-] + K_{mu} K_1'/f_{HCO_3} a_H}{k_e E}. \qquad (8)$$

K_{mu} may thus be also obtained indirectly from CO_2 output experiments with mixtures of $NaHCO_3$ and buffer in the pH range in which the uptake is too small for accurate measurement. The value of

$f_{HCO_3} a_H$ is obtained by means of equations (24) or (25) of Roughton & Booth (1946), and R_{eo} calculated as therein described.* $[HCO_3^-]/R_{eo}$ is then plotted against $[HCO_3^-]$, and the value of $K_{mu}K_1'/f_{HCO_3}a_H$ is given as before by the intercept on the $[HCO_3^-]$ axis, whereas the cotangent of the angle made with the latter gives $k_e E$. In this treatment the concentration of the actual substrate, H_2CO_3, disappears, which is an advantage since our estimate of it depends on the value of K which is only approximately known.

We have done several experiments of this kind with the chloroform enzyme solution and at 0·0025 to 0·02 M-$NaHCO_3$ both in M/20–M/50 cacodylate buffer, pH 6·2, and in M/30 phosphate buffer, pH 6·3. The results, however, show a rather wide scatter owing mainly to the diffusion corrections being so large in this range, not only for the enzymic but also for the non-enzymic reactions. Drawing the best lines through the data gives, however, values of $K_{mu} = 0{\cdot}009$ (± about 40 %). With $NaHCO_3$ concentrations of 0·01–0·03 M in M/40 phosphate buffer, pH 7·0 (29 p.p.m. of chloroform enzyme), the reaction rates are much slower, and yield the value of 0·009 to within ± 0·001, as in Fig. 2. On the whole, it seems safe to assume therefore that K_{mu} is independent of pH over the range 6·0–10·0.

That K_{mu} is also independent of partial purification is shown by comparison of the squares with the circles in Fig. 2, the squares representing results obtained in M/24 veronal, pH 8·0, 0° with blood solution (7·1 p.p.m. of red cells) in place of the chloroform enzyme solution. On this basis we might expect that the value of K_{mu} would be the same in pure solutions of carbonic anhydrase, but the lesser stability of the latter makes it very difficult to obtain accurate enough measurements by the manometric technique. Since this work was completed, however, Kiese (1941) has reported values of K_{mu} for a highly purified preparation from horse red cells. After the usual ethanol-chloroform procedure, the enzyme was further purified by fractional precipitation with ammonium sulphate and dialysis. His value at pH 7·4 and 1° was only 0·0012, i.e. about one-seventh of our value and was furthermore affected by pH, rising to 0·0022 at pH 9·3. Kiese knew of, and took, some of the precautions in estimation listed above and by Roughton & Booth (1946), but it is not clear that he allowed for them all—a matter of great importance with pure solutions. Apart from this, we have no explanation of the difference from our results other than the special purity of his enzyme solutions. Leiner (1943), working with a highly purified enzyme preparation in presence of peptone as stabilizer at pH 7·39, 0°, gives some data on uptake rates at several CO_2 pressures. His results only permit an approximate calculation of K_{mu}, but the value we find from them, namely 0·0075 (± 20 %), is in the same range as our own values of K_{mu} rather than the values given by Kiese.

Experimental proof that the enzyme catalyzes both phases of the reaction equally, i.e. that $k_{eu} = k_{eo} = k_e$. Kiese & Hastings (1940*b*) have shown, as expected, that at equilibrium carbonic anhydrase catalyzes both CO_2 output and CO_2 uptake equally: in their determinations, however, no allowance was made either for the limiting effect of diffusion, which was probably but slight in many instances, or for the change in activity of the enzyme during the manometric shaking, which may sometimes have been quite large. We have made two special tests with chloroform enzyme solution in phosphate buffer, under conditions where the diffusion correction is not more than 15 %.

(*a*) At pH 7·3:

(i) CO_2 uptake experiment:

$[CO_2] = 0{\cdot}00143$ M; enzyme = 57 p.p.m.

$R_{eu} = 3{\cdot}7 \times 10^{-3}$ mol. CO_2/l./sec.

Then by equation (1)

$$\frac{k_{eu}E \times 0{\cdot}00143}{0{\cdot}00143 + 0{\cdot}009} = 3{\cdot}7 \times 10^{-5},$$

whence $k_{eu}E = 2{\cdot}70 \times 10^{-4}$.

(ii) CO_2 output experiment:

$[HCO_3^-] = 0{\cdot}02$ M; enzyme = 28·6 p.p.m.

$f_{HCO_3}a_H = 4{\cdot}25 \times 10^{-8}$; $R_{eo} = 3{\cdot}2 \times 10^{-5}$ mol. CO_2/l./sec.

Then by equation (7)

$$\frac{k_{eo}E \times 0{\cdot}02}{0{\cdot}02 + 0{\cdot}009 \times 2{\cdot}6 \times 10^{-7}/4{\cdot}25 \times 10^{-8}} = 3{\cdot}2 \times 10^{-5},$$

whence $k_{eo}E = 1{\cdot}2 \times 10^{-4}$.

Thus

($k_{eu}E$ in CO_2 uptake experiment)/($k_{eo}E$ in CO_2 output experiment) = 2·24

as against a value of 2·0 for the ratio of the enzyme concentration in the two experiments.

(*b*) At pH 7·5:

In a similar pair of experiments we had (i) $[CO_2] = 0{\cdot}00149$ M, enzyme = 14·3 p.p.m. and (ii) $[HCO_3^-] = 0{\cdot}032$ M, enzyme concentration = 28·5 p.p.m. From the values of R_{eu} and R_{eo} we found

($k_{eu}E$ in CO_2 uptake experiment)/($k_{eo}E$ in CO_2 output experiment) = 0·465,

whereas (enzyme concentration in CO_2 uptake experiment)/(enzyme concentration in CO_2 output experiment) was equal to 0·5.

* In the allowance for diffusion by equation (18) of the adjoining paper, a value for K_{mu} is assumed. A 100 % variation in the assumed value of K_{mu}, however, makes a change of only about 10 % in the calculated value of R_{eo}; hence it seems fair provisionally to assume $K_{mu} = 0{\cdot}009$ in this calculation and use the results so obtained to test equation (8).

Thus in these two tests we find that $k_{eu}=k_{eo}=k_e$ to within ± 10 % on the average. This is well within experimental error.

EFFECT OF BUFFER CONCENTRATION

Table 1 summarizes results for the four main buffer systems used. The values of k_eE listed in the last column are calculated as in the examples just given, a constant value for K_{mu} of 0·009 M at 0° being assumed throughout. With the two more alkaline buffers, veronal and dimethylglyoxaline, k_eE is constant within the experimental error (± 10 %) over the range 0·015 to 0·08 M-buffer concentration, and its actual values therefore probably depend only on the effect of pH on the activity of the enzyme. A similar result was found in preliminary experiments with two other alkaline buffers, namely, succinimide and allantoin. As regards the effect of phosphate, Exp. D of Table 1 clearly shows a depressing effect at concentrations of total buffer $\geqslant$ 0·048 M (i.e. $[HPO_4^=] > 0{\cdot}036$ M). Part, but not nearly all, of this depression is due to the lowered pH at higher concentrations of phosphate (see below). At lower concentrations of $HPO_4^=$ (i.e. 0·024 M and less), Exps. B and C show practical constancy, though in Exp. D the value at 0·0096 M appears to be definitely below those at 0·015 and 0·024 M-total phosphate. Exp. D was, however, done at an early stage in the development of the technique: in Exp. C, which was carried out nearly at the end of the research, the low range of phosphate concentration was carefully reinvestigated, and the value of k_eE was found to be constant within experimental error. This constancy would best be confirmed by working at concentrations below 0·0096 M-phosphate, but the total CO_2 uptake then becomes too small for accurate measurement.

The effect of cacodylate concentration has proved very hard to establish satisfactorily. Of several difficulties the worst is that in CO_2 output experiments below pH 6·4 the correction for the limiting effect of diffusion becomes very large, i.e. 50 % or more, and correspondingly doubtful. The values given in Exp. A of Table 1 seem, as they stand, to show a significant activating effect of this buffer at concentrations above 0·048 M. A similar result was found in two other experiments, but in view of the experimental and computative uncertainties, the activation by cacodylate cannot be regarded as definitely established.

In the standard boat method of Meldrum & Roughton (1933) for measuring anhydrase activity, M/5 $NaHCO_3$ is mixed with an equal volume of M/5 phosphate buffer, pH 6·8, ± enzyme, and the rate of CO_2 output followed. According to Table 1, an inhibition of the order of 40 % is produced by this concentration of phosphate, and it is therefore necessary, in comparing the activity of the enzyme from different sources or under different conditions (e.g. added 'activators'), to be sure that the inhibitory effect of the phosphate is the same in the various cases. This precaution has not been taken in most previous work (see later).

Table 1. *Effect of buffer concentration on activity of enzyme*

Exp.	Buffer: Type	Buffer: Acid/salt	Buffer: Conc.	pH	k_eE
	Substrate 0·005 M-HCO_3^-. Enzyme 47·6 p.p.m.				
A	Cacodylate	1·0	0·096	6·18	$1{\cdot}79\times10^{-4}$
			0·048	6·21	$1{\cdot}24\times10^{-4}$
			0·024	6·23	$1{\cdot}43\times10^{-4}$
			0·012	6·24	$1{\cdot}07\times10^{-4}$
	Extrapolation		0·0		$1{\cdot}10\times10^{-4}$
	Substrate 0·010 M-HCO_3^-. Enzyme 28·6 p.p.m.				
B	Phosphate	1·0	0·048	6·93	$8{\cdot}55\times10^{-5}$
			0·024	7·01	$8{\cdot}45\times10^{-5}$
	Substrate 0·00149 M-CO_2. Enzyme 14·3 p.p.m.				
C	Phosphate	0·36	0·024	7·48	$5{\cdot}77\times10^{-5}$
			0·0154	7·52	$6{\cdot}28\times10^{-5}$
			0·0096	7·56	$5{\cdot}6\times10^{-5}$
D	Phosphate	0·33	0·096	7·37	$4{\cdot}48\times10^{-5}$
			0·048	7·44	$5{\cdot}45\times10^{-5}$
			0·024	7·51	$7{\cdot}0\times10^{-5}$
			0·0154	7·55	$7{\cdot}5\times10^{-5}$
			0·0096	7·59	$6{\cdot}19\times10^{-5}$
E	Veronal	0·33	0·044	8·21	$9{\cdot}1\times10^{-4}$
			0·015	8·25	$8{\cdot}0\times10^{-4}$
F	Veronal	0·20	0·083	8·48	$9{\cdot}5\times10^{-5}$
			0·050	8·51	$9{\cdot}5\times10^{-4}$
			0·024	8·54	$10{\cdot}2\times10^{-4}$
G	Dimethylglyoxaline	3·0	0·038	8·41	$8{\cdot}4\times10^{-5}$
			0·019	8·38	$7{\cdot}65\times10^{-5}$
H	Dimethylglyoxaline	0·33	0·067	9·40	$12{\cdot}3\times10^{-5}$
			0·029	9·36	$12{\cdot}0\times10^{-5}$

THE EFFECT OF pH

Fig. 3 summarizes our complete data as to the effect of pH upon anhydrase activity in the various buffer solutions for a standard concentration of 14·3 p.p.m. of crude chloroform enzyme in each case. The output method was used between pH 5·5 and 7·5, and the uptake method between pH 6·5 and 10. The size of the rectangles denotes the probable experimental error (± 10 %), whilst the enclosed letter indicates the nature of the buffer used. The concentration of the latter was below the limit at which, according to Table 1, there is a significant effect of the buffer on the observed activity (e.g. 0·03 M in case of phosphate).

The results between pH 6·2 and 9·0 are clear-cut and satisfactory. There is a fairly broad minimum

between pH 6·2 and 6·7, and thereafter the curve rises almost linearly with pH. In pH zones in which different buffers overlap (namely pH 6·2–6·5 for cacodylate and phosphate, pH 7·5–8·0 for phosphate and veronal, pH 8·2–8·7 for veronal and dimethylglyoxaline, and pH 8·8–9·0 for dimethylglyoxaline and pyrophosphate) there is good agreement whichever buffer is used, in spite of the great diversity in the chemical nature of the various buffers, especially compounds (e.g. ammonia and glycine). Succinimide does not suffer from any of these disadvantages, but it is unstable in solution and the three points obtained with this buffer between pH 9·0 and 10·0 are presented therefore only tentatively. They do, however, suggest a steady rise of the curve above pH 9·0 rather than a plateau.

The shape of the curve given in Fig. 3 differs from that put forward by Kiese (1941), who reports that

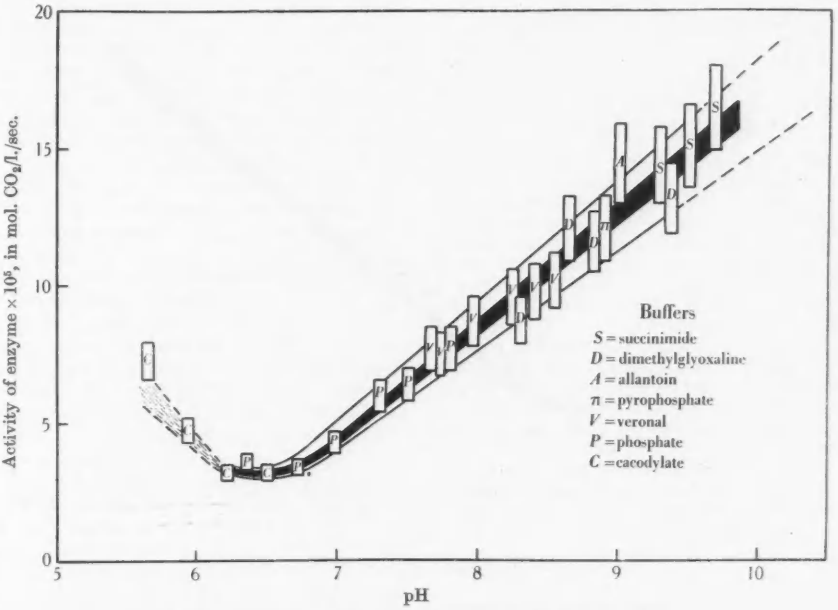

Fig. 3. Activity-pH curve for chloroform enzyme preparation.

in their ionizing group. This suggests that the relationship shown in Fig. 3 is dependent only upon pH and is not clouded by activating or inhibitory effects of the buffers.

Below pH 6·0 the curve seems to rise again, though it must be admitted that (i) there is still some suspicion that even at low concentrations cacodylate may have an activating effect, and we have not yet found any other convenient and non-inhibitory buffer with which to check the results in this range, (ii) as already mentioned the corrections for the limiting effect of diffusion become uncomfortably large.

There is likewise a dearth of satisfactory buffers above pH 9. The usual systems inhibit the enzyme (e.g. phenate, borate, and carbonate), catalyze the non-enzymic reaction too greatly (e.g. borate), or react independently with CO_2 to form carbamino

the pH curve has a plateau below pH 6·0 and also above pH 9·0, whilst between these limits it has the same shape as the titration curve of a weak acid of pK = 7·2. We had reached the same conclusion at an earlier date as Kiese (1941) courteously acknowledged. The differences of our present results from those of Kiese are, we believe, partly due to more accurate methods of observation and calculation, and partly to the fact that Kiese assumed that K_{mu} increases with pH, whereas, according to our finding, K_{mu} is independent of pH.

We have also made some study of the effect of pH on the enzyme in other degrees of purification. Fig. 4 shows a much smaller series of experiments upon the effect of pH on the anhydrase activity of ox red cell solutions. All the points except that at pH 8·5 fall to within 12% upon the same pH activity curve as in Fig. 3. The same concordance

in the overlapping regions of phosphate and cacodylate buffer, phosphate and veronal buffers, is again noted.

Towards the end of the research we carried out a few experiments upon an enzyme preparation highly purified by the method of Keilin & Mann, to whom we are indebted for this sample. Of the six pH 7·50. The latter buffer solution, it will be recalled, has apparently no inhibitory effect. The results with chloride and nitrate show the same diminution in activity for the three cations Na^+, K^+ and NH_4^+; the anions on the other hand have marked specific effects, the series running $SO_4^- <$ acetate$^-$, CL^-, $\ll Br^-$, NO_3^-, I^-. Except possibly in

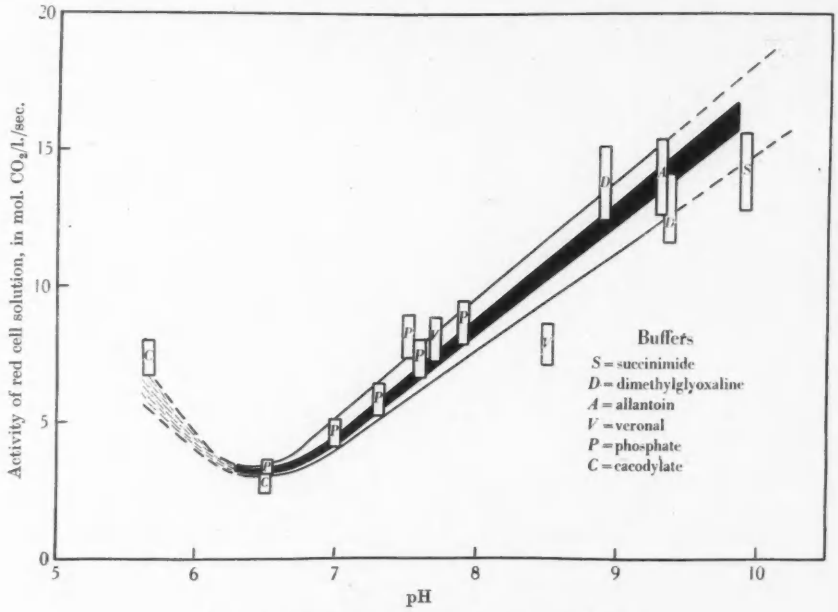

Fig. 4. Activity-pH curve for lyzed ox red cell solution.

points obtained, one fell right off the previous pH-activity curves of Figs. 3 and 4, but the remaining points were all fairly close. Two different concentrations of the enzyme were used in all cases, and a fair-to-good proportionality between concentration and activity was observed except in two instances, namely, at pH 6·2 and 8·2, wherein results similar to those of Fig. 1*B* were found.

Our provisional conclusion is that purification is without significant effect on the pH-activity curve of carbonic anhydrase, as has also been found for yeast saccharase by Euler, Josephson & Myrbäck (1924).

EFFECT OF CERTAIN SALTS

The study of the effect of salts is only preliminary and limited in scope. Table 2 gives the effect of adding a small series of neutral salts (analytical grade purity, final concentration mostly 0·024 M) on the activity of 14·3 p.p.m. crude chloroform enzyme solution in 0·024 M-potassium phosphate, the case of sulphate, the effects observed are much larger than could be accounted for by the changes in pH produced by adding the salts.

Table 2. *Effect of some neutral salts on anhydrase in* 0·024 M-*phosphate*, pH 7·5

Salt	Molarity	Activity in salt + phosphate / Activity in phosphate
NH_4Cl	0·024	0·70
KCl	0·024	0·61
NaCl	0·024	0·69
NaCl	0·096	0·33
KBr	0·024	0·196
KI	0·024	0·172
KNO_3	0·024	0·182
$NaNO_3$	0·024	0·177
K_2SO_4	0·072	0·565
Na acetate	0·024	0·60
Na cacodylate	0·048	0·97

The results at the two NaCl concentrations conform with a reversible reaction of Cl^- with the enzyme, i.e. $E + Cl^- \rightleftharpoons E.Cl^-$, the complex being

inactive and having at pH 7·5 a dissociation constant of about 0·045. The affinity of the enzyme for Cl^- is found to decrease markedly with increase of pH, the inhibition by 0·05M-Cl^- being barely detectable in veronal and dimethylglyoxaline buffers above pH 8·4. On this account it has been possible to utilize the dimethylglyoxaline-dimethylglyoxaline hydrochloride buffer systems over almost their whole pH range, but in the case of the veronal series with their lower pH range we had to prepare the buffer systems mostly by addition of diethylbarbituric acid to the sodium salt rather than by the usual practice of adding HCl.

We have no data yet on the action of Cl^- below pH 7·5, but in the case of acetate, the inhibitory effect of which at pH 7·5 is about the same as that of Cl^-, we have some preliminary comparisons of the activity of the enzyme at pH 5·6 with that in cacodylate at the same pH. In 0·06M-acetate the activity is about 0·4 of that in cacodylate, i.e. a degree of inhibition agreeing roughly with that found in 0·06M-acetate at pH 7·5. There is obvious scope for much fuller study of the effects of salts along the quantitative lines worked out by Myrbäck (1926) for amylase and by Mann & Woolf (1930) for fumarase.

The salts of other carboxylic acids would presumably inhibit like acetate, but data are lacking. In early tests on two dicarboxylic acid buffers at pH 5·6, namely, 0·06M-maleate and 0·036M-phthalate, we found activities of only 0·04 and 0·09 respectively of that of cacodylate at this pH.

The fact that 0·048M-Na cacodylate (see bottom row of Table 2) has no appreciable effect gives support to the not quite proven conclusion that moderate concentrations of cacodylate are neither inhibitory nor activating.

In carbonate solutions, the activity of the enzyme is less than in other solutions of the same pH according both to Kiese (1941) and to our own independent results, in which quite large inhibition was observed. This has a bearing on the method of Philpot for estimating anhydrase activity, in which the time is taken for the pH of a solution containing 0·025M-Na_2CO_3 in 0·017M-$NaHCO_3$ to drop from 10·5 to 7·0 at 0° when CO_2 is bubbled through the solution ± enzyme. It would be expected, therefore, that the enzyme would be inhibited under these conditions. From the comparisons with the boat method given in the original paper (Philpot & Philpot, 1936), together with the present data on the effect of substrate concentration, etc., we calculate that the degree of inhibition is of the order of 90% as compared with the activities found in the dilute buffer solutions used in Fig. 3. The importance of being sure that so large a degree of inhibition remains constant when the effect of activators and other factors is studied, is even more obvious than in the case of the standard boat method (see above).

In Brinkman's (1933) method, CO_2 solution is added to a bicarbonate-carbonate solution of pH 9·1, and the time taken for the pH to drop to the turning point of phenol red is measured at 0°. We have no quantitative data as to the degree of inhibition in this case, but there is reason to believe it still to be large, though less than in the Philpot technique.

In view of these drawbacks to the existing CO_2-indicator methods of measuring anhydrase activity, we have carried out some preliminary experiments with veronal buffer, which is non-inhibitory, in place of carbonate.

SOME RESULTS WITH A CO_2 VERONAL-INDICATOR METHOD

Veronal buffer (3 ml. of 0·022M-veronal in 0·22M-Na salt, pH 7·95), three drops of bromothymol blue, and 2·3 ml. of distilled water (or 0·3 ml. enzyme solution + 2·0 ml. distilled water) are mixed in a 15 ml. weighing bottle which is stoppered and placed in ice for at least 15 min. Ice cold water (5 ml.) saturated with CO_2 (= 0·071M-CO_2 as determined by Van Slyke analysis) is then added anaerobically from an all-glass syringe with a long nozzle and the time is observed for the pH to drop to pH 6·3, with the aid of a matching standard of bromothymol blue in phosphate buffer at pH 6·3. By placing the nozzle below the buffer solution the CO_2 can be injected and the solutions all mixed in less than a second without bubbling or loss of CO_2. The end-point is chosen at a pH where there is a sharp change in the colour of the indicator and the buffer power of the solution is very low, so that the pH is changing rapidly.

In absence of enzyme the following times were noted: 89, 88, 93; mean 90 sec. In presence of enzyme 64, 63, 68, 62; mean 64 sec. The same enzyme solution was used as in Fig. 3 and its final concentration was identical, namely, 14·3 p.p.m.

The validity of the method was first checked by calculating approximately the value of k_u (the velocity constant of the uncatalyzed reaction $CO_2 + H_2O \rightarrow H_2CO_3$) from the blank tests without enzyme. Allowing a period of 1 sec. for the time of mixing of the CO_2 and buffer solutions, we have

At $t = 1$ sec., ['veronate' ion] $= 0·022 \times 3/10·5 = 0·0063$M,
$[HCO_3^-]$ = zero,
$[CO_2] = 0·071 \times 5/10·5 = 0·0337$M.

At $t = 90$ sec., ['veronate' ion] $= 0·00015$M,
$[HCO_3^-] = 0·00615$M,
$[CO_2] = 0·0276$M,

since $[HCO_3^-]$ at $t = 90 - [HCO_3^-]$ at $t = 1$ equals [veronate] at $t = 1 -$ [veronate] at $t = 90$ equals $[CO_2]$ at $t = 1 - [CO_2]$ at $t = 90$.

The average $[CO_2]$ during the experiment $= 0·0307$M.

So k_u is approximately equal to $[HCO_3^-]$ formed/{[average CO_2] × (90 − 1)}

$$= 0{\cdot}00615/(0{\cdot}0307 \times 89)$$
$$= 0{\cdot}00225 \text{ mol./l./sec.}$$

This value must, however, be reduced by about 2 % to allow for the catalytic effect of the veronate ion on the $CO_2 + H_2O \rightarrow H_2CO_3$ reaction. The final value, i.e. 0·0022, agrees well with the accepted value for k_u at 0°, namely, 0·0021.

The enzymic activity is next calculated as follows. The overall rate of enzymic and blank reactions together is proportional to 1/(64 − 1) sec., and the rate of the blank reaction alone to 1/(90 − 1) sec.

The ratio of the intrinsic enzymic rate to the blank rate therefore

$$= (1/63 - 1/89) \div 1/89 = 0{\cdot}413.$$

So that the rate of the enzymic hydration of CO_2

$$= 0{\cdot}413 \times 0{\cdot}00225 \times 0{\cdot}0307$$
$$= 2{\cdot}85 \times 10^{-5} \text{ mol. } CO_2\text{/l./sec.}$$

From equation (1)

$$\frac{k_{eu}[\text{average } CO_2]\,E}{[\text{average } CO_2] + K_{mu}} = 2{\cdot}85 \times 10^{-5}$$

or
$$k_{eu}E = \frac{2{\cdot}85 \times (0{\cdot}0307 + 0{\cdot}009)}{0{\cdot}0307} \times 10^{-5}$$

$= 3{\cdot}7 \times 10^{-5}$ mol. CO_2/l./sec. over the pH range covered in the experiment, i.e. 7·9–6·3.

According to Fig. 3, the manometric value of $k_{eu}E$ at the beginning of the experiment (pH 7·9) = 8·0 and at the end (pH 6·3) = 3·0, and it can be shown by numerical approximation that the average value during the run should be about equal to the actual value at pH 7·35, i.e. 5·5.

The figure for $k_{eu}E$ obtained by the all-liquid method thus comes out at about 70 % of that expected from the manometric data. About one-third of the *c.* 30 % deficit may be accounted for by slight inhibitory action of the indicator (Kiese & Hastings, 1940*b*) and the remainder (20 %) is not large in view of the experimental uncertainties of the two methods (± 10 %) and the approximate character of the calculations. The degree of agreement in these preliminary tests suggests on the whole that any special effects of adsorption and/or inactivation at the gas/liquid interface in the manometric technique have been mainly controlled by our procedure.

Further tests under a wider variety of conditions are obviously desirable, but it is hoped that the detailed working out as given above may be of use in further research in this field, particularly in the study of those activators which, according to Leiner (1941), are so specially effective in the enzymic hydration of CO_2, as investigated by the all-liquid method.

THE MELDRUM & ROUGHTON ENZYME UNIT

Meldrum & Roughton (1933) defined the enzyme unit provisionally as the amount which, when dissolved in 2 ml. of phosphate solution (M/10 Na_2HPO_4 in M/10 KH_2PO_4) and mixed with 2 ml. of bicarbonate solution (M/5 $NaHCO_3$ in M/50 NaOH), halves the time for evolution of the second quarter of total CO_2 at 15°. This unit was useful in the early work on the enzyme, but fails to satisfy present-day requirements for the following reasons. (1) No allowance is made for the progressive inactivation of the enzyme during the shaking: even at 0° this is considerable and variable. (2) At the concentrations of phosphate used there is an inhibition of the order of 40 %, which may also be variable with different enzyme preparations. (3) No corrections for diffusion are made. At 15° they would be higher than at 0°, and therefore more sensitive to changes in the conditions of shaking, etc. (4) No account is taken of the possibility of a response curve of the type shown in Fig. 1*B*.

On these grounds it has not seemed feasible to translate the Meldrum & Roughton unit to the absolute units of this paper, and we do not recommend the continued use of the former unit except for relatively crude work. For accurate estimations of carbonic anhydrase activity the most suitable manometric procedure would seem to be the measurement of the rate of CO_2 uptake at 0° by dilute phosphate (> 0·02 M) or chloride-free veronal buffer in the range pH 7·5–8·5, with graded amounts of enzyme, by the technique and mode of calculation described in this paper.

DISCUSSION

The question of activators. Most of the data in this paper are on crude and semi-purified enzyme solutions in the presence of one of the limited series of buffers which have little or no effect on the activity of the enzyme, in so far as this can be established by varying the buffer concentration over the range 0·01–0·10 M. No studies were made with activators, since our work was done before their discovery. According, however, to Leiner (1941) and Main & Locke (1941) the effect of activators on red cell solutions is small, if appreciable, presumably because the carbonic anhydrase is already fully 'activated' by accompanying substances derived from the haemolyzed cells. Our data on the latter may therefore well be independent of activator effects. With the semi-purified enzyme solutions, the effect of activators is stated to be much more pronounced, but in any detailed study thereof it is clearly necessary to make full allowance for the effect of pH, buffers and the other factors dealt with

above. The methods and results developed in this and the accompanying paper should therefore be of service in, and form a basis for, more exact and comprehensive research on the effects of activators in semi-purified and highly purified enzyme solutions.

The actual existence of activators was for some time vigorously debated in Germany between Leiner and Kiese, the latter of whom was unable to detect any activation in many of the instances brought forward by Leiner. Owing to the war some of the relevant publications may well have escaped our notice but in the most recent work of Kiese (1942) to reach us clear proof is advanced as to the ability of glutathione to activate both the enzymic hydration and dehydration of CO_2. This led Kiese to withdraw from his earlier denial of the existence of activators, but he concluded, and we believe with reason, that 'obviously the conditions for activation are not yet sufficiently clear'. As pointed out in a previous section, in most of the work which has been done on activators the enzyme preparations are already considerably inhibited by the medium in which the estimation is made. Many so-called 'activations' may indeed be no more than reversible restoration of activity lost through (*a*) the inhibitory effect of the buffer itself, or of traces of impurities present in it, in the distilled water or on the walls of the glass apparatus; (*b*) adsorption at interfaces and surface denaturation. These possibilities are obviously of particular importance in the case of highly purified enzyme solutions, wherein enzyme concentrations of the order of M/300,000,000 are dealt with and the protective substances present in the less purified preparations have been removed. In such work it is essential not to omit *any* of the precautions listed by Roughton & Booth (1946), and it is equally important to verify that the relation between enzyme concentration and activity is linear and not of the form shown in Fig. 1*B*, otherwise entirely misleading results would be obtained. In none of the previous work known to us on activators have all these points been covered, and for that reason quantitative knowledge on this subject seems open to question, though it must be admitted that the effects so far described appear too large for the qualitative demonstration of activators to be doubted. As suggested elsewhere (Roughton, 1943) it would be very desirable to adopt the rapid flow methods of Hartridge & Roughton (1923) to the estimation of carbonic anhydrase activity so that far higher concentrations of the purified enzyme could be used, thus reducing the chance of activity loss through the factors mentioned above. With this technique it should also be possible to work at body temperature which is outside the range of the manometric method even with diffusion corrections. Data derived from such estimations are obviously desirable for a more accurate appraisal of the role of the enzyme in physiological processes.

The activity of carbonic anhydrase in the intact red cell. In 1931, a year before carbonic anhydrase was discovered, Dirken & Mook showed that, when a suspension of red cells was mixed with serum saturated with CO_2, equilibrium as regards CO_2 and bicarbonate was reached to within 10% in about 0·2 sec. In such experiments a considerable amount of CO_2 hydration occurs inside the red cell, and since this process, as well as all the others involved, is accomplished in 0·2 sec. there is no escape from the conclusion that the intact red cell has a high anhydrase activity. This result was confirmed by the manometric experiments of Booth (1938) which show clearly the importance also of accessibility factors, namely the chloride-bicarbonate interchange and the rapid supply and removal of H^+ ions by buffers inside the red cell. Further interesting evidence, of a qualitative kind, was available after Mann & Keilin's (1940) discovery of the inhibitory action of sulphanilamide. It was easy to show manometrically that the rates of CO_2-uptake and output by red cell suspensions are greatly reduced in the presence of sulphanilamide (Roughton, Dill, Darling, Graybiel, Knehr & Talbott, 1941; Davenport, 1941; see also the later experiments of Fegler, 1944). The same result was likewise obtained by Keilin & Mann (1941) and by Jacobs & Stewart (1942) using less direct, though very elegant, methods of observation.

Thus there is no doubt whatever of a high anhydrase activity inside the red cell, though its magnitude is still uncertain. Owing to the limiting effect of diffusion and of the 'accessibility factors' studied by Booth, no direct measurement has yet been made of the activity of the intact red cells, though such estimation is much needed for appraising the exact role of carbonic anhydrase in speeding up the exchange of CO_2 between the blood and the lungs or tissues. In default thereof, Roughton (1935) has tentatively assumed that the activity remains proportional to concentration up to the high values present in the red cell and, from the activities of the red cells when laked, diluted and measured with the standard Meldrum & Roughton method, he estimated that the anhydrase of the red cells was capable of magnifying 1500-fold the rate of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ at 38°. No proper allowance could, however, be made at the time for the effects of pH, and of concentrations of substrate, phosphate and chloride on the activity of the enzyme, and with the new data on these factors given in this paper the figure of 1500-fold needs to be replaced (tentatively) by one of 7500-fold (Roughton *et al.* 1941). A further increase might be necessary for the effect of activating factors in the cell. Roughton (1935) also calculated that the

reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ needs to be magnified 150 times in rate within the blood if the CO_2 in the lung capillaries is to come to within 10% of equilibrium with the CO_2 in the alveolar air in 1 sec., i.e. the average time hitherto assumed to be spent by the blood in the lung capillaries. This calculation, however, requires modification in light of the more recent data on (i) the velocity constants of the $CO_2 + H_2O \rightleftharpoons H_2CO_3$ reaction and the true first ionization constant of carbonic acid at 38° (Roughton, 1943, Table 1); (ii) the average time spent by the blood in the human lung capillaries, which, according to Roughton's (1945) theoretical estimations, ranges from 0·75 sec. at rest to 0·35 sec. in hard work. On this basis, magnifications ranging from 300- to 650-fold would be required in the circulating blood, and if the calculated magnification of 7500-fold in the intact red cell is a correct figure it follows that in normal blood the enzyme is present in 12- to 25-fold excess of minimum requirements. These revised figures accord rather more closely with the results of Roughton *et al.* (1941) on the effects of sulphanilamide on CO_2 output in man at rest and during exercise than did the previous figures which these authors used.

SUMMARY

1. Manometric determinations are reported on the effect of substrate concentration, pH and neutral salts on the activity of semi-purified and crude carbonic anhydrase at 0°. The activities are calculated in mol. CO_2/l. absorbed or evolved per second, and allowance is made for (i) the limiting effect of diffusion, (ii) progressive denaturation caused by the shaking, (iii) inhibitory effects of the buffers and of impurities present in the solution.

2. The Michaelis constant is found to be independent of pH: its value at 0° = 0·009 M-CO_2 ± 0·001.

3. In the pH range in which CO_2 uptake and output can both be measured, carbonic anhydrase catalyzes each phase equally.

4. The effect of pH was studied over the range pH 5·6–10·0 with the aid of four main buffer systems, namely, cacodylate, phosphate, veronal and dimethylglyoxaline, the inhibitory effect of these buffers being inappreciable at concentrations below 0·05 M. The pH curve shows a minimum between 6·0 and 7·0, and is still rising at pH 10·0. The shape is therefore probably not that of the ionization curve of a univalent weak acid, as had been previously reported.

5. The anions of many neutral salts were found to be inhibitory at pH 7·4 when present in concentrations of 0·02–0·1 M, the series running $SO_4^= < Cl^- \ll Br^-$, NO_3^-, I^-. The anions of most carboxylic acids are also inhibitory.

6. The enzyme is strongly inhibited in the bicarbonate-carbonate solutions used in the Philpot method of estimation, and an alternative all-liquid method using veronal buffer is therefore proposed.

7. The relation of the present work to that of other investigators on activators of carbonic anhydrase is discussed.

8. The quantitative activity of the enzyme in the red cell *in vivo* is reconsidered in the light of the new data reported in this paper.

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